Implication des bactéries du genre Arthrobacter dans la coloration de surface des fromages à pâte molle et croûte lavée

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Implication des bactéries du genre *Arthrobacter* dans la coloration de surface des fromages à pâte molle et croûte lavée

Soutenance le 26 septembre 2014 devant le jury composé de :

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SOMMAIRE

SOMMAIRE (CONTINU)

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RESUME

Les fromages à pâte molle et à croûte lavée, caractérisés par leur couleur jaune-orange-rouge en surface, sont des produits laitiers de grande consommation en Europe. La couleur de la surface semble essentiellement due à la présence de caroténoïdes, en combinaison avec d'autres pigments de nature inconnue, produits par la microflore du fromage en cours d'affinage. *Arthrobacter* sp. est l'un des principaux micro-organismes qui se développent sur la surface des fromages, particulièrement sur les fromages à croûte lavée, et sa pigmentation jaune est supposée contribuer à la couleur globale de ces produits laitiers.

Les micro-organismes produisant des pigments sont aisément trouvés dans la nature. Aujourd'hui, ceux-ci suscitent un grand intérêt dans de nombreuses disciplines scientifiques et les applications se sont élargies dans l'industrie en raison de leurs avantages biotechnologiques. Comme la tendance actuelle mondiale pointe vers l'utilisation de produits biosourcés et biodégradables, l'exigence d'ingrédients naturels, en particulier les colorants naturels, augmente de jour en jour. La première partie de cette thèse met en évidence le rôle crucial des micro-organismes comme sources potentielles de production de pigments naturels, en s'appuyant sur un grand nombre de travaux de recherche liés aux pigments biosynthétisés par des micro-organismes, travaux publiés au cours des 10 dernières années par des entreprises privées ou des laboratoires universitaires, avec un accent mis sur les pigments prévoyant une application alimentaire.

Certaines parties de cette thèse comprennent l'examen de la possibilité de produire des biomasses pigmentées d'*Arthrobacter* sp. comme nouvelles sources de colorants alimentaires, le genre *Arthrobacter* étant un groupe de bactéries métaboliquement très polyvalent et très courant dans la nature. Par ailleurs, les aspects bénéfiques de l'espèce *Arthrobacter* sp. dans l'industrie laitière au sens large sont également abordés.

Compte tenu de l'importance du genre *Arthrobacter* sp. dans les fromages à croûte lavée, ainsi que dans les produits laitiers économiquement importants, l'objectif de la recherche décrite dans cette thèse est d'étudier l'implication de ces bactéries, en particulier l'espèce *Arthrobacter arilaitensis*, dans la coloration de ces fromages selon plusieurs aspects couvrant (i) la diversité de la production de pigment (s) parmi les souches, (ii) la cinétique de la synthèse du/des pigment(s), (iii) la détermination des caractéristiques chimiques des pigments, (iv) la caractérisation de la pigmentation par

levures de désacidification, sur la production de pigments et le développement de couleur.

Parmi les 14 souches d'*Arthrobacter arilaitensis* étudiées, deux groupes peuvent être formés en fonction de leur capacité de production de caroténoïdes, les souches produisant des caroténoïdes et celles non pigmentées dans les conditions de l'étude. La cinétique de synthèse de caroténoïdes par ces souches indique une pigmentation associée à la croissance. La diversité de la concentration de pigments parmi les souches productrices de caroténoïdes est faible, et similaire aux caractéristiques de pigmentation déterminées par spectrocolorimétrie quantitative. L'analyse HPLC-PDA-APCI-MS de pigments extraits de deux souches représentatives a révélé huit caroténoïdes différents dont la C_{50} décaprénoxanthine, le principal caroténoïde accumulé.

Des changements dans le développement de la couleur de souches d'*A. arilaitensis* sous l'influence des facteurs physiques, chimiques et biologiques ont été obtenus et analysés par spectrocolorimétrie quantitative. Trois groupes ont été formés en fonction du comportement de coloration affecté par la lumière : positivement sensible, négativement sensible et non sensible à la lumière. Le pH acide et une forte concentration de sel ont montré un effet d'inhibition sur la pigmentation d'une souche représentative d'*A. arilaitensis*. En combinaison avec le pH et la teneur en NaCl, les levures de désacidification du caillé fromage ont aussi eu un impact sur la production des pigments d'*A. arilaitensis*. La valeur moyenne la plus élevée de saturation de la couleur a été observée sur les milieux désacidifiés par *Debaryomyces hansenii* à pH 7,0, montrant un jaune intense.

A l'issue de ces recherches, les résultats obtenus fournissent des informations utiles aux fabricants de fromages et de produits laitiers lors de la sélection de souches d'*A. arilaitensis*, dans l'objectif d'optimiser la coloration de surface sans usage de colorant ajouté.

Sutthiwong, N. 2014. Implication of bacteria of the genus Arthrobacter in the surface coloration of the smear-ripened soft cheeses. Academic dissertation. University of Reunion Island, Faculty of Sciences and Technologies.

ABSTRACT

Smear-ripened soft cheeses, characterized by their orange-red color on rind, are dairy products widely consumed in Europe. The surface color is due essentially to carotenoids, in combination with other pigments, produced by the cheese microflora during ripening. *Arthrobacter* sp. is one of the major microorganisms occurred on the surface of cheeses, particularly in smear-ripened cheeses, where it is assumed to be responsible for yellow pigmentation of the cheese rind because of its characteristic overall color and its involvement at the different stages of cheese ripening.

Pigment-producing microorganisms are commonly found in the nature. Nowadays, pigment-producing microorganisms have been increasing of interest in many scientific disciplines and applications have broadened in the industry because of their biotechnological advantages. As the present trend entirely the world is shifting toward the use of eco and biodegradable products, the requirement for natural ingredients, especially natural colorants, is increasing day by day. The first part of this thesis highlights the crucial role of microorganisms as potential sources of natural pigment production by reviewing a large number of research works related to pigments biosynthesized by microorganisms which were published over the past 10 years by private companies or academic laboratories, with an emphasis on pigments providing for the application in foods.

Since the genus *Arthrobacter* is a group of metabolically versatile bacteria which widely distributed in nature, some parts of this thesis include the review presenting the possibility to produce pigmented *Arthrobacter* sp. biomasses as novel sources of food colorants; furthermore, the beneficial aspects of *Arthrobacter* sp. and their promising significances in the dairy industry are also addressed.

Considering the significance of Arthrobacter sp. in smear-ripened cheeses, the economically important dairy products, the aim of research described in this thesis is to investigate the implication of this bacterium, particularly *Arthrobacter arilaitensis*, in the coloration of these cheeses in several aspects covering (i) diversity of pigment production among strains, (ii) kinetic of pigment synthesis, (iii) identification of chemical characteristic of pigments, (iv) colorimetric characterization of pigmentation, and (v) influences of environment i.e. light, pH, NaCl and deacidifying yeasts on the production and the color development of pigments.

Among 14 strains of *Arthrobacter arilaitensis* studied, two groups depending on their ability of carotenoid production could be divided, carotenoid-producing and non-pigmented strains. A growth-associated pigmentation probably applied to indicate the kinetic of carotenoid synthesis by these strains. The diversity of pigment concentration among the carotenoid-producing strains was low, related to the characteristics of pigmentation determined by quantitative spectrocolorimetry. The HPLC-PDA-APCI-MS analysis of extracted pigments of a representative strains revealed 8 different carotenoids showing C50 decaprenoxanthin as the major accumulated carotenoids.

Changes in the color development of *A.arilaitensis* strains under the influences of physical, chemical and biological factors were obtained through spectrocolorymetry. Three groups depending on a coloration behavior affected by light were illustrated e.g. positively sensitive, negatively sensitive and not sensitive to light. The acidic pH and high concentration of salt showed the efficiency inhibited effect on pigmentation of a representative strain of carotenoid-producing *A. arilaitensis*. In combination of pH and NaCl, deacidifying yeasts were obviously related to the pigment production of *A. arilaitensis*. The highest average value of color saturation were observed on the studied media deacidified by *Debaryomyces hansenii* at pH 7.0, displaying intense yellow.

Following this research, the results provide useful information to manufacturers of cheese and dairy products in the selection of *Arthrobacter arilaitensis* strains, with the aim of optimizing the surface color without using additional colorants.

CHAPITRE 1

Pigments microbiens

Les colorants sont utilisés pour de nombreux produits allant de la nourriture aux vêtements et textiles. Ils déterminent l'attrait du produit, mais aussi l'acceptabilité des consommateurs. Depuis de nombreuses années, la prise de conscience exprimée pour la sécurité des personnes et la protection de l'environnement a éveillé un nouvel enthousiasme pour les sources naturelles de couleurs. Cette circonstance est fortement due à deux raisons principales, *i*), de nombreux colorants synthétiques, qui ont largement été utilisés dans la fabrication des divers produits, révèlent plusieurs effets dangereux et *ii*) la tendance actuelle où tout le monde se déplace vers l'utilisation des marchandises éco-compatibles et biodégradables (Venil *et al*., 2009; Dufossé, 2006). Par ces motifs, la nécessité de colorants naturels ne cesse d'augmenter. Les colorants naturels peuvent être généralement obtenus à partir de deux sources principales, les plantes et les micro-organismes. Alors que les pigments accessibles à partir des plantes ont plusieurs difficultés, en particulier la non-disponibilité tout au long de l'année, les micro-organismes semblent être des sources plus avantageuses. Plusieurs facteurs renforcent l'intérêt des pigments microbiens, par exemple, la vitesse de croissance des micro-organismes et la faisabilité du développement de bioprocédés qui créent une production de pigments naturels indépendants de la saison et des conditions géographiques, ainsi que les propriétés bénéfiques de pigments microbiens au-delà de l'aspect couleur, e.g. antioxydant (Pandey *et al*., 2007; Mojib *et al*., 2010).

Les microorganismes producteurs de pigments, y compris les bactéries, les moisissures, les levures et les algues sont très répandus dans la nature. De nos jours, certains micro-organismes ont été utilisés avec succès comme sources pour la production de pigment commercial par exemple *Monascus* sp. pour pigment rouge, *Penicillium oxalicum* pour le rouge arpink, *Xanthophyllomyces dendrorhous* pour l'astaxanthine, *Ashbya gossypii* pour la riboflavine, et *Blaskeslea trisposa* pour le β-carotène (Sutthiwong *et al*., 2013; Dufossé, 2006). Cependant, avec la préférence croissante des consommateurs résultant de la demande sur le marché des ingrédients naturels, la production et l'application de pigments microbiens parmi les colorants naturels ont été constamment sérieusement étudiées par un grand nombre

de scientifiques. La tâche de découvrir des pigments microbiens appropriés pour l'utilisation est difficile parce que des pigments ne doivent pas seulement fournir une couleur désirée, mais aussi être sans danger pour la consommation. En conséquence, un micro-organisme produisant des pigments idéaux doit satisfaire à certains critères, i) la possibilité d'utiliser une variété de sources de C et N, ii) la tolérance aux conditions de croissance (par exemple, le pH, le température, la concentration en nutriments), iii) fournir la couleur attendue avec un rendement raisonnable, iv) produit non-toxique et microorganisme non-pathogène , et v) extraction simple des pigments à partir de la biomasse cellulaire s'il n'est pas excrété (Zhou *et al.*, 2009; Hailei *et al*., 2012). Plusieurs nouveaux micro-organismes produisant les pigments tels que certains groupes de bactéries (la bactérie productrice de caroténoïdes *Streptomyces* sp., la productrice de prodigiosine *Serratia* sp., la productrice de pigment bleu intense *Pantoea* sp.) et de champignons filamenteux (le producteur de physcion *Alternaria* sp., le producteur de flavoglaucin *Microsporum* sp.) sont actuellement émergents dans l'objectif de démontrer leur potentiel en tant qu'usines cellulaires microbiennes pour la production de pigments impliquant diverses applications industrielles, en particulier pour l'industrie alimentaire (Dufossé *et al*., 2014; Dharmaraj *et al*., 2009).

Aujourd'hui, la biotechnologie joue probablement un rôle crucial pour une grande production des colorants naturels en raison des recherches approfondies qui ont été faites pour développer des bioprocédés pour produire des pigments de divers micro-organismes; cependant, les limitations technologiques sont encore le principal obstacle à la capitalisation commerciale. En mettant l'accent sur les mécanismes métaboliques, grâce à une approche multidisciplinaire impliquant les aspects de la microbiologie, la biochimie, la chimie et de l'ingénierie, une percée majeure dans la production de pigments microbiens pourrait être atteinte dans un proche avenir. Par cette stratégie, les micro-organismes pourraient être créés pour produire des pigments à haut rendement par l'insertion de gènes codant pour le pigment, même les micro-organismes qui ne produisent pas de pigments naturels.

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Article 1

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En raison de l'augmentation mondiale de la fabrication d'aliments transformés et les demandes des consommateurs vis-à-vis des ingrédients alimentaires naturels, le marché des colorants naturels destinés à l'alimentation est en progression constante. Actuellement, la grande majorité des colorants alimentaires naturels autorisés dans l'Union européenne et les Etats-Unis sont dérivés de l'extraction des pigments à partir de matières premières obtenues à partir de la floraison de plantes du royaume Plantae. La production de nombreux colorants naturels d'origine végétale existants a un inconvénient lié à la dépendance de l'approvisionnement en matières premières, qui est influencée par les conditions agro-climatiques – de plus, leur profil chimique peut varier d'un lot à l'autre. En outre, la plupart des pigments provenant des sources contemporaines sont sensibles à la chaleur, à la lumière et à l'oxygène, et certains peuvent même changer leur couleur en réponse aux changements de pH. Ces inconvénients peuvent être surmontés par la mise au point et le développement de pigments microbiens de qualité alimentaire qui sont susceptibles de réduire le coût de production, ce qui conduit à une source de colorants alimentaires naturels moins cher. La production de colorants naturels par la fermentation microbienne a un certain nombre d'avantages par rapport aux systèmes à base de plantes telles que des rendements plus élevés dans une période de temps plus courte et pas de variations saisonnières.

Cet article met l'accent sur les travaux de recherche liés à la production de pigments d'origine microbienne publiés au cours des 10 dernières années par des entreprises privées ou des laboratoires académiques, en mettant l'accent sur les pigments pour un usage alimentaire.

12

Production of Biocolors

Nuthathai Sutthiwong, Yanis Caro, Philippe Laurent, Mireille Fouillaud, Alain Valla, and Laurent Dufossé

CONTENTS

12.1 Introduction

Nature is rich in colors (minerals, plants, microalgae, etc.) and pigment-producing microorganisms (fungi, yeasts, bacteria) are quite common (Figure 12.1). Currently, the vast majority of the natural food colorants permitted in the European Union and the United States are derived by extraction of the pigments from raw materials obtained from the flowering plants of the kingdom Plantae. The production of many existing natural colorants of plant origin has a disadvantage of dependence on the supply of raw materials, which are influenced by agro-climatic conditions—in addition, their chemical profile may vary from batch to batch. Moreover, many of the pigments derived from the contemporary sources are sensitive to heat, light, and oxygen, and some may even change their color in response to pH changes as in case of anthocyanins. Until recently, problem of color loss and stability in products could be easily tackled by using synthetic pigments, such as azo dyes, originally derived from coal tar. This view has changed over the last 5 years as

FIGURE 12.1
Spontaneous development of pigmented microorganisms at the surface of nutritive agar Petri dish.

concerns about possible adverse health effects of synthetic colors have grown (Southampton study, McCann et al., 2007).

Among the molecules produced by microorganisms are carotenoids, melanins, flavins, phenazines, quinones, bacteriochlorophylls, and more specifically monascins, violacein, or indigo (Figure 12.2) (Dufossé, 2004; Kerr, 2000; Plonka and Grabacka, 2006). The success of any pigment produced by fermentation depends upon its acceptability in the market, regulatory approval, and the size of the capital investment required to bring

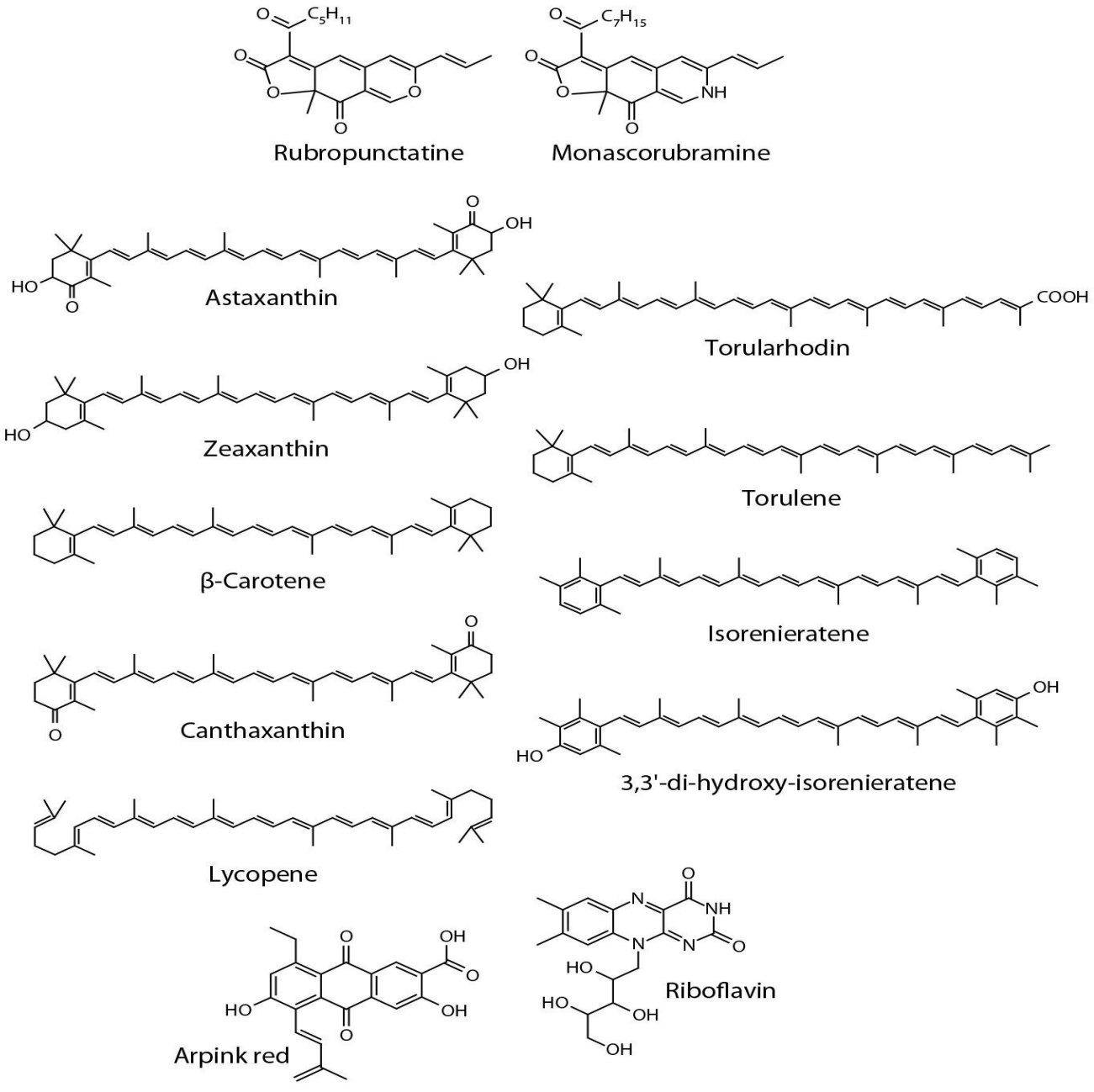

FIGURE 12.2
Some microbial food-grade pigments.

the product to market. A few years ago, some expressed doubts about the successful commercialization of fermentation-derived food-grade or cosmetic-grade pigments because of the high capital investment requirements for fermentation facilities and the extensive and lengthy toxicity studies required by regulatory agencies. Public perception of biotechnology-derived products also had to be taken into account. Nowadays, some fermentative food-grade pigments are on the market: *Monascus* pigments, astaxanthin from *Xanthophyllomyces dendrorhous*, arpink red from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, and β-carotene from *Blakeslea trispora*. The successful marketing of pigments derived from algae or extracted from plants, both as a food color and as a nutritional supplement, reflects the presence and importance of niche markets in which consumers are willing to pay a premium for "all natural ingredients."

Colors can serve as the primary identification of food and are also a protective measure against the consumption of spoiled food. Colors of foods create physiological and psychological expectations and attitudes that are developed by experience, tradition, education, and environment: "We inevitably eat with our eyes."

The controversial topic of "synthetic dyes in food" has been discussed for many years and was amplified in 2007 with the Southampton study (Mc Cann et al., 2007; Schab and Trinh, 2004) and its transcription in a legal frame (i.e., the use of warning labels in Europe about a hyperactivity link for products containing any of the Southampton colors is mandatory since July 2010). The scrutiny and negative assessment of synthetic food dyes by the modern consumer have given rise to a strong interest in natural coloring alternatives. Some companies decided to "color food with food," using mainly plant extracts or pigments from plants, for example, red from paprika, beetroots, berries, or tomato; yellow from saffron or marigold; orange from annatto; and green from leafy vegetables.

Penetration of the fermentation-derived ingredients into the food and cosmetic industries is increasing year after year. Examples could be taken from the following fields: thickening or gelling agents (xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium glutamate), flavor compounds (gamma-decalactone, diacetyl, methyl-ketones), acidulants (lactic acid, citric acid), etc. Efforts have been made in order to reduce the production costs of fermentation pigments compared to those of synthetic pigments or pigments extracted from natural sources (Dufossé, 2006). Innovations will improve the economy of pigment production by isolating new or creating better microorganisms, by improving the processes.

This chapter focuses on research works related to this field published over the past 10 years by private companies or academic laboratories, with an emphasis on pigments for food use. As recently described by our group, there is "a long way from the Petri dish to the market place" and thus to the product on store shelves.

12.2 *Monascus* Pigments, an Old Story for Asians

12.2.1 *Monascus* Pigment

Monascus is cultivated on solid medium in Asian countries to produce a red colorant named "Anka" used as a food ingredient. In a Chinese medical book on herbs published in the first century, this term "ang-kak" or "red mold rice" was first mentioned. Red mold rice has been used as a food colorant or spice in cooking. In 1884, a purple mold was isolated on potato and linseed cakes and was named it *Monascus ruber*. This ascomycete was so named as it has only one polyspored ascus. Then in 1895, another strain was isolated from the red mold rice obtained from the market in Java, Indonesia. This fungus was named *Monascus purpureus*. Then several other species were isolated around the world. *Monascus* is often encountered in oriental foods, especially in Southern China, Japan, and Southeastern Asia. Currently, more than 50 patents have been issued in Japan, the United States, France, and Germany, concerning the use of *Monascus* pigments for food. Annual consumption of *Monascus* pigments in Japan moved from 100 tons in 1981 to 600 tons at the end of the 1990s and was valued at $1.5 million. New food applications, like the coloration of processed meats (sausage, hams), marine products like fish paste, surimi, and tomato ketchup were described (Blanc et al., 1994).

12.2.2 Fungal Metabolites

The main metabolites produced by *Monascus* are polyketides, which are formed by the condensation of one acetylcoA with one or more malonylcoA with a simultaneous decarboxylation as in the case of lipidic synthesis. The metabolites mainly consist of the pigments, monacolins, and, under certain conditions, of a mycotoxin (Juzlova et al., 1996).

Monascus pigments are a group of fungal metabolites called azaphilones, which have similar molecular structures as well as similar chemical properties. Two molecular structures of the *Monascus* pigments are shown on Figure 12.2. Ankaflavin and monascine are yellow pigments, rubropunctatine and monascorubrine are orange, and rubropunctamine and monascorubramine are purple. The same color exists in two molecular structures differing in the length of the aliphatic chain. These pigments are produced mainly in the cell-bound state.

They have low water solubility, are sensitive to heat, are unstable in the pH range of 2–10, and fade with light. A number of methods have been patented in order to make water-soluble pigments. The principle is the substitution of the replaceable oxygen in monascorubrine or rubropunctatine by nitrogen of the amino group of various compounds such as amino acids, peptides, and proteins, changing the color from orange to purple. *Monascus* pigments can be reduced and oxidized and can react with other products, especially amino

acids, to form various derivative products sometimes called the complexed pigments. Glutamyl-monascorubrine and glutamyl-rubropunctatine were isolated from the broth of a submerged culture.

Stability of the pigments is affected by acidity, temperature, light, oxygen, water activity, and time. It was shown that these pigments added to sausages or canned pâté remained stable for 3 months' storage at 4°C, while their stability ranged from 92% to 98%. Thus, the main patents have focused on the solubilization, the stability, and the extraction in solution of pigments. The pigments can easily react with amino group-containing compounds in the medium such as proteins, amino acids, and nucleic acids, to form water-soluble pigments.

A series of hypocholesteremic agents have been isolated from *Monascus* and named monacolin J, K, and L. These polyketides were first isolated from cultures of *Penicillium citrinum* and they can inhibit specifically the enzyme controlling the rate of cholesterol biosynthesis. They are currently used in China in traditional and modern medicine.

Antibacterial properties of *Monascus* were first mentioned in 1977. The so-called monascidin A was effective against *Bacillus*, *Streptococcus*, and *Pseudomonas*. It was shown that this molecule was citrinin and its production by various *Monascus* species was studied using different culture media and conditions.

12.2.3 Methods of Production

12.2.3.1 Submerged Fermentations

Considerable contradiction exists in the published works as to the best carbon source for red pigment production in liquid cultures. Traditionally cultured on breads and rice, *Monascus* grows on every amylaceous substrate. *Monascus* grows quite well on starch, dextrines, glucose, maltose, and fructose. High production of pigments was achieved using glucose and maltose. The nitrogen source seems to have more importance than the carbon source and ammonium, and peptones as nitrogen sources gave superior growth and pigment concentrations compared to nitrate. The best results were obtained using glucose and histidine. The carbon/nitrogen ratio was also shown to be important: at a value close to 50 g/g, growth would then be favored, while in the region of 7–9 g/g, pigmentation would be favored.

12.2.3.2 Solid-State Fermentations

The classical Chinese method consists of inoculating steamed rice grains spread on big trays with a strain of *Monascus anka* and incubating in an aerated and temperature-controlled room for 20 days (Babitha et al., 2007). In these types of cultures, moisture content, oxygen, and carbon dioxide levels in the gas environment, as well as cereal medium composition, are the most important parameters to control.

Moisture content is a very important parameter. Red pigments were produced in plastic bags containing rice grains. It was observed that pigmentation occurred only at a relatively low initial moisture level (26%–32%). Initial substrate moisture content regulated pigmentation as it was found that glucoamylase activity increased along with a rise in the initial substrate moisture content. Therefore, at high moisture content, as high enzyme activity was produced, glucose was rapidly liberated in high amounts (120 g/L) with ethanol formation, which inhibited pigmentation.

Thus it was confirmed that solid culture was superior to liquid culture for red pigment production by *M. purpureus*. This result has been attributed to the derepression of pigment synthesis in solid systems due to the diffusion of intracellular pigments into the surrounding solid matrix. In submerged fermentation, the pigments normally remain in the mycelium due to their low solubility in the usually acidic medium.

Levels of oxygen and carbon dioxide in the gas environment influence pigment production significantly while affecting growth to a lesser extent in solid-state culture. With *M. purpureus* on rice, maximum pigment yields were observed at 0.5×10^5 Pa of oxygen partial pressure in closed pressure vessels. However, high carbon dioxide partial pressures progressively inhibited pigment production, with complete inhibition at 10^5 Pa. In a closed aeration system with a packed-bed fermentor, oxygen partial pressures ranging from 0.05 to 0.5×10^5 Pa at constant carbon dioxide partial pressures of 0.02×10^5 Pa gave high pigment yields with a maximum at 0.5×10^5 Pa of oxygen, whereas lower carbon dioxide partial pressures at constant oxygen partial pressures of 0.21×10^5 Pa gave higher pigment yields. Maximum oxygen uptake and carbon dioxide production rates were observed at 70–90 and 60–80 h, respectively, depending on the gas environment. Respiratory quotients were close to 1.0 except at 0.05×10^5 Pa of oxygen and 0.02×10^5 Pa of carbon dioxide partial pressures.

When studying various cereal media, it was shown that the best results were obtained using "mantou" meal (yeast-fermented wheat meal).

12.2.4 Methods to Control Mycotoxin Production

In order to chemically identify the so-called monascidin A discussed by some Chinese scientists in their papers as a component suitable for the preservation of food, this compound was isolated and chemical investigations using mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) were undertaken. Monascidin A was characterized as citrinin (Xu et al., 2003). Thus, in order to avoid the production of this toxin, various strains were screened in order to see if all were toxinogenic, and it was shown that in the species of *Monascus* available in public collections, nontoxigenic strains were obtainable.

Another way to avoid the production of citrinin can be through controlling the biosynthesis of the metabolite. To control the biosynthesis,

the metabolic pathway has to be investigated. The metabolic pathway is the same for citrinin and the pigment: the polyketide pathway in which condensation of acetates and malonates occurred. In the case of the pigment, there is at the end of the pathway an esterification of a fatty acid on the chromophore to obtain the colored molecules. Several modifications of the culture conditions are possible in order to increase the pigment production or reduce the citrinin one: addition of fatty acids, change of the nitrogen source. Adding fatty acids to the medium was effective in favoring the synthesis of pigment, but the citrinin production remained unchanged.

The final modification of the culture conditions was the replacement of glutamic acid by other amino acids. *M. ruber* was cultivated in a liquid medium containing glucose and various amino acids, and histidine was found to be the most effective nitrogen source regarding citrinin production inhibition. When the pathway of histidine assimilation was investigated, it was shown that during its catabolism, one molecule of hydrogen peroxide was produced per molecule of consumed histidine and it is known that peroxidases can destroy citrinin in the presence of hydrogen peroxide. So the production of citrinin can be avoided by control of the medium especially by the selection of a suitable amino acid, usually histidine.

Despite the enormous economic potential of *Monascus* pigment, it does not lead to a commercial exploitation in the Western world, mainly because of ignorance and also because of reluctance to change from food public agencies. Indeed, these agencies do not approve *Monascus* pigments for use in the food industry, although they do appear to be nontoxic if correctly used. Thus, even though species of *Monascus* have been consumed in the Far East for many years, this does not help the pigment to gain approval in the European Union or the United States.

12.2.5 *Monascus*-Like Pigments from Nontoxigenic *Penicillium* Species

A screening for novel producers of *Monascus*-like pigments was conducted among ascomycetous fungi belonging to *Penicillium* species that are not reported to produce citrinin or any other known mycotoxins (Mapari et al., 2005, 2008). Monascorubrin, xanthomonasin A, and threonine derivatives of rubropunctatin were identified in the extract of *Penicillium aculeatum* IBT 14263, and monascorubrin was identified in the extract of *Penicillium pinophilum* IBT 13104. None of the tested *Penicillium* extracts showed the presence of citrinin. Thus, the present study brought out two novel promising sources of yellow, orange, and purple-red *Monascus*-like food pigments in the species of Penicillia that do not produce citrinin and opened the door to look for several more new promising sources of natural food colorants in the species of Penicillia.

12.3 Microbial Anthraquinones

12.3.1 Arpink Red from *P. oxalicum*

Many patents from Ascolor s.r.o. (Czech Republic) relate to a new fungus strain having the properties to produce a red colorant that can be applied in the food and cosmetic industries (WO9950434, CZ285721, EP1070136, US6340586) (Sardaryan et al., 2004). The strain *P. oxalicum* var. Armeniaca CCM 8242, obtained from soil, produces a chromophore of the anthraquinone type (Figure 12.2). Some strains of the same species are effective as biological control agents, for example, reduction of the incidence of *Fusarium* wilt of tomato under glasshouse and field conditions. Others have been described for the production of milk-clotting enzyme.

The cultivation of the fungus in liquid broth requires carbohydrates (such as sucrose, molasses), nitrogen (corn extract, yeast autolysate, or extract), zinc sulfate, and magnesium sulfate. The optimum conditions for performing the microbiological synthesis are pH value 5.6–6.2 and temperature 27°C–29°C. On the second day of incubation, a red colorant is released into the broth, increasing up to 1.5–2.0 g/L of broth after 3–4 days of incubation. After biosynthesis of the red colorant is completed, the liquid from the broth is filtered or centrifuged for being separated from the biomass. The liquid is then acidified to pH 3.0–2.5 to precipitate the colorant. The precipitate is dissolved in ethyl alcohol and filtered. Following the removal of alcohol, the colorant in the crystalline form is obtained, i.e., dark red powder.

The colorant gives a raspberry-red color in aqueous solution, stable at pH over 3.5. Neutral solutions are stable even after 30 min of boiling and color shade does not change in relation with pH.

Many toxicological data are available on this red pigment: acute oral toxicity in mice, 90-day subchronical toxicological study, acute dermal irritation/corrosion, acute eye irritation/corrosion, antitumor effectiveness, micronucleus test in mice, AMES test (*Salmonella typhimurium* reverse mutation assay), estimation of antibiotic activity, and results of estimation of five mycotoxins. A new patent on arpink red was filed in 2001 with claims of anticancer effects of the anthraquinone derivatives and applications within the food and pharmaceutical fields.

After evaluating all the materials provided by the company Ascolor Biotech s.r.o., the Codex Alimentarius Commission (Rotterdam meeting, March 11–15, 2002) made the following statement: "there will not be any objections to use the red colouring matter Arpink Red" in meat products in the amount up to 100 mg/kg, meat and meat product analogues in the amount up to 100 mg/kg, nonalcoholic drinks in the amount up to 100 mg/kg, alcoholic drinks in the amount up to 200 mg/kg, milk products in the amount up to 150 mg/kg, ice creams in the amount up to 150 mg/kg, and confectionery in the amount up to 300 mg/kg.

12.3.2 Other Microbial Anthraquinones

Anthraquinoid molecules are derivatives of "9,10-anthraquinone" (which is also called 9,10-anthracenedione or 9,10-dioxoanthracene, i.e., an aromatic organic compound with formula $C_{14}H_8O_2$ and whose ketone groups are on the central ring B). In general, for each anthraquinoid molecule, there are eight possible hydrogens that can be substituted. Anthraquinoid pigments are a class of naturally occurring pigments that are mainly distributed in nature, especially from insects or tinctorial plants (e.g., *Rubia, Galium, Rheum*). Many anthraquinones are colored and they provide the most important red dyes and lakes used in artistic paintings. They are relatively stable and light-fast and they give a bright color. These properties led to their use for selected applications at present, such as in textile dyeing, printing applications, and cosmetic formulation (e.g., alizarin pigment from madder), and for some of them in food manufacturing (e.g., red carminic acid from cochineal as coloring agent for beverages or processed meat). Industrially, microbial anthraquinone pigments were first isolated from cultures of *P. oxalicum var. Armeniaca* as mentioned earlier (case of arpink red). However, the extraction, isolation, and characterization of other microbial anthraquinones have more recently been reported in the literature from some filamentous fungi with different shades such as red, bronze, maroon, and yellow (Figure 12.3).

For example, the anthraquinone pigment emodin (orange) has been isolated and identified from cultures of both *P. citrinum* and *Penicillium islandicum* (Duran et al., 2002; Frisvad, 1989; Mapari et al., 2009). The red pigment produced by strain of *Isaria farinosa* was recently elucidated as a chromophore of the anthraquinone type (Velmurugan et al., 2010). Similarly, the red pigment produced by *Paecilomyces sinclairii*, which was beforehand discovered but uncharacterized (Cho et al., 2002), is certainly of identical chemical nature according to Velmurugan et al. (2010), i.e., an amino group linked to an anthraquinone structure. Some strains of *Eurotium* could produce other microbial anthraquinones such as physcion (yellow), flavoglaucin (yellow), auroglaucin (orange), and erythroglaucin (red). From both strains of *Aspergillus*, i.e., *A. sulphureus* and *A. westerdijkiae*, viopurpurin (purple), which is an anthraquinone pigment, has been isolated and identified. In the same way, *Aspergillus glaucus* has been identified as a possible source of emodin (orange) and physcion (yellow) pigments. Strains of *Emericella purpurea* could synthesize both azaphilone and anthraquinone pigments such as epurpurins A–C (yellow) (Hideyuki et al., 1996). Some red anthraquinone pigments have also been isolated from cultures of *Fusarium oxysporum* and *Fusarium moniliforme*; moreover, the dyeing potential of the red pigment produced by *F. oxysporum* was assessed for woolen materials by Nagia and El-Mohamedy (2007). *Curvularia lunata* was also known to produce different anthraquinone pigments like chrysophanol (red), helminthosporin (maroon), and cynodontin (bronze). The catenarin (red) pigment has been isolated and identified from cultures of different *Drechslera*, i.e., from *D. teres, D. graminea, D. tritici-repentis, D. phlei*, and *D. dictyoides*. From *Drechslera avenae*,

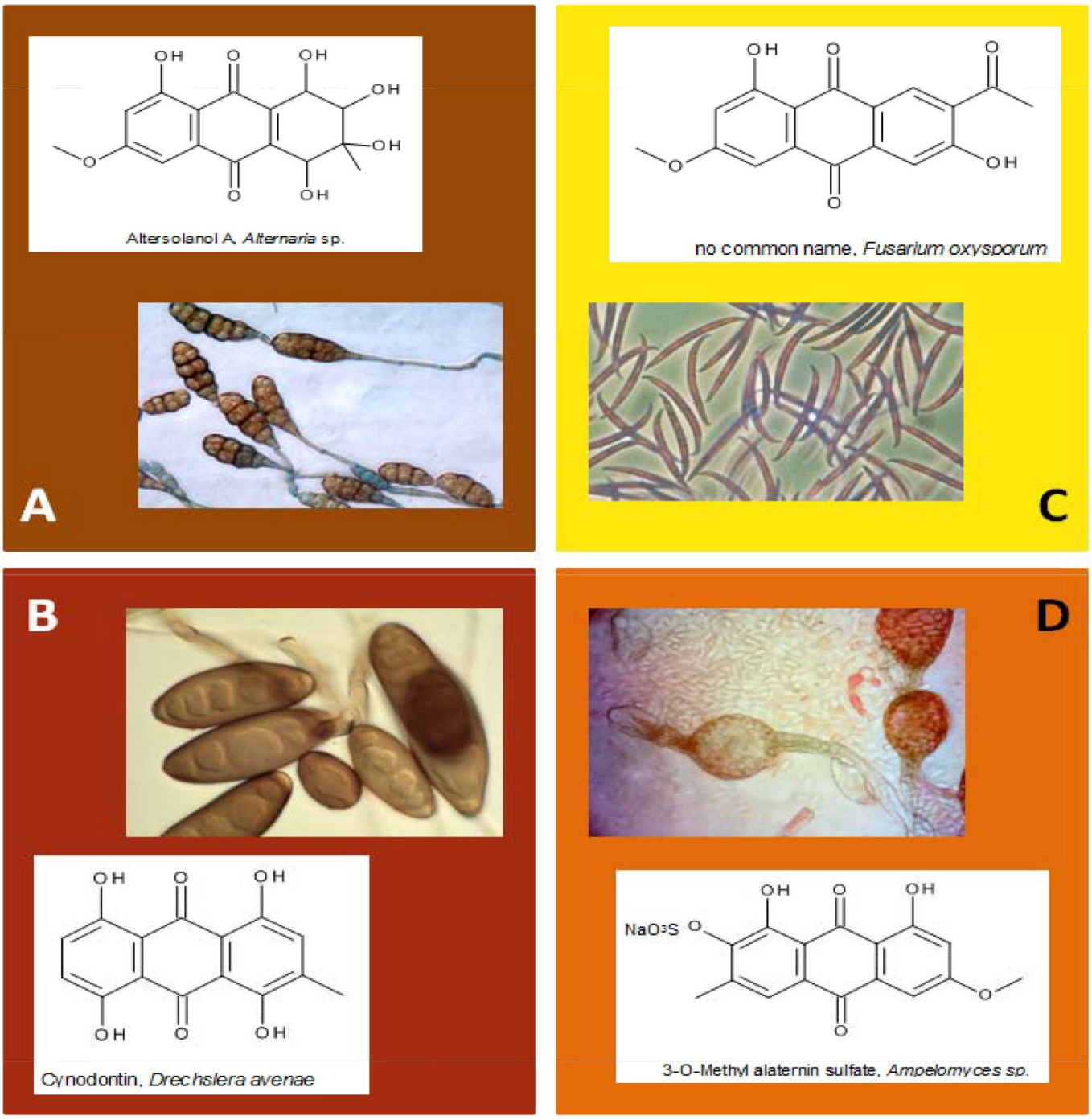

FIGURE 12.3
Some anthraquinones from fungal origin: (a) maroon, (b) bronze, (c) yellow, (d) red-orange.

more particularly, both anthraquinone pigments have been isolated and identified: cynodontin (bronze) and helminthosporin (maroon) (Duran et al., 2002; Engström et al., 1993). Other anthraquinone pigments like averythrin have been isolated and identified from a culture of *Herpotrichia rhodosticta*, which is a member of the genus *Pyrenochaeta* (Van Eljk and Roeijmans, 1984). Some strains of *Dermocybe* sp. were also well known to produce physcion (yellow) and dermocybin pigments.

Like the anthraquinone pigment isolated and identified from cultures of *I. farinosa*, the solubility in water of anthraquinone pigments is generally excellent, with some exceptions (e.g., rhein). For some anthraquinone pigments, the color of the molecule changed according to variations in pH of the aqueous solution; for example, it is the case for the microbial pigment produced both by *I. farinosa* and by *Pa. sinclairii* (yellow to red in acidic solution, violet in neutral solution, and pale violet in alkaline solution) and also for alizarin and purpurin (yellow to red [acid]; red to violet [alkaline]). Concerning stability, such as carminic acid from cochineal, which is one of the few natural and water-soluble colorants that resist degradation with time, it was shown that microbial anthraquinone pigments have good stability to heat, chemical oxidation, light, and oxygen. Most of them, like the *I. farinosa* pigment, were relatively stable at 60°C and below and subjected to steaming and sunlight exposure showed no change of color.

As for all secondary metabolites, it is frequently reported that the pigment production is influenced by many factors. Among them, the morphological stages of the fungi, the composition of the culture medium, and the cultural conditions. However, such factors as temperature, initial pH of the medium, agitation speed, nitrogen and carbon concentrations, osmolarity, light exposition, and age of the inoculum seem to have different impacts on the yields and the production rates of the pigments, depending on the strains involved. As an example, in the glaucus group, the nutritive needs, as well as the nature of the pigments produced, can change if the fungus develops the cleistothecial form or the conidial stage. The initial pH of the culture seems to have not much influence on the rate of the pigment production by *Eurotium cristatum* (*As. glaucus* group) (Anke et al., 1980). The temperature and the carbon availability (glucose) influence the overall yields, in so far as the growth is increased, but does not enhance the rate of pigment-specific production (mg pigment/g dried mycelium). The addition of salt (NaCl) and malt extract is necessary to obtain the high osmotic pressure essential for the growth of several strains of the group and therefore to the pigment production. However, a high salt concentration (2 M) decreases the yield of pigment (per g of dried mycelium). On the other hand, the addition of yeast extract, which enhances the nitrogen content, strongly increases the mycelium production but drastically decreases the pigment synthesis. The negative effect of a high N content on the pigment production has been noticed in several cases. Moreover, it was inferred from the results that for all the species of the *As. glaucus* group studied, the mycelium contains considerably larger amounts of pigments than the culture filtrates. The level of pigment production by *Penicillium* sp. rises with agitation speed up to 200 rpm and is reduced thereafter (Gunasekaran and Poorniammal, 2008). The inoculum age for an optimal production of pigment was about 4 days. Some experiments on the suitable aeration rates for pigment production by *Pa. sinclairii* showed that the maximum yield was achieved with 3.5 vvm, whereas the maximum biomass concentration

was obtained with an aeration rate of 1.5 vvm (Cho et al., 2002). It seems that this parameter had complex effects, due to the relation between the mycelium morphology (highly branched or highly vacuolated) and the quality of oxygen transfers. The optimization of pigment production in filamentous fungi could therefore be based on some common concepts but requires specific adaptations depending on the producing strains.

12.4 Riboflavin, a Yellow Food Colorant but Also the Vitamin B_2

Riboflavin (vitamin B_2) has a variety of applications as a yellow food colorant. Its use is permitted in most countries. Applications include dressings, sherbet, beverages, instant desserts, ice creams, tablets, and other products. Riboflavin has a special affinity for cereal-based products, but its use in these applications is somewhat limited due to its slight odor and naturally bitter taste. There are numerous microorganisms that produce riboflavin fermentatively. Riboflavin fermentation (Burgess et al., 2009) could be classified into three categories: weak overproducers (100 mg/L or less, e.g., *Clostridium acetobutylicum*), moderate overproducers (up to 600 mg/L, e.g., yeasts such as *Candida guilliermondii* or *Debaryomyces subglobosus*), and strong overproducers (over 1 g/L, e.g., the fungi *Eremothecium ashbyii* and *A. gossypii*) (Kapralek, 1962).

12.5 Fluorescent Pink from the Red Microalga *Porphyridium*, Phycobiliproteins

The red microalga genus *Porphyridium* is a source of biochemicals possessing nutritional and therapeutical values. These biochemicals include polysaccharides (having anti-inflammatory and antiviral properties), long-chain polyunsaturated fatty acids, carotenoids such as zeaxanthin, and fluorescent phycobiliproteins (Bermejo Roman et al., 2002).

The phycobiliproteins are accessory photosynthetic pigments, aggregated in the cell as phycobilisomes, which are attached to the thylakoid membrane of the chloroplast. The red phycobiliproteins, phycoerythrin, and the blue phycobiliprotein, phycocyanin, are soluble in water and can serve as natural colorants in food, cosmetics, and pharmaceuticals. Chemically, the phycobiliproteins are built up of chromophores—the bilins—which are open-chain tetrapyrroles, covalently linked via thioether bonds to an apoprotein.

The microalgae are cultured in bioreactors under solar or artificial light in the presence of carbon dioxide and salts. The bioreactors could be closed systems made of polyethylene sleeves rather than open pools. Optimal conditions for pigment production are low to medium light intensity and medium temperatures (20°C–30°C) (Kathiresan et al., 2007).

Pigment extraction is achieved by cell breakage, extraction into water or buffered solution, and centrifugation to separate out the filtrate. The filtrate may then be partly purified and sterilized by microfiltration and spray dried or lyophilized.

Porphyridium sp. is the source of a fluorescent pink color. The main *Porphyridium* sp. phycobiliproteins are β-phycoerythrin and β-phycoerythrin. Maximum absorbance of a 1% solution of B-phycoerythrin in a 1 cm cuvette is at 545 nm, and the fluorescence emission peak is at 575 nm. Batch culture of *Porphyridium* sp. outdoors yields approximately 200 mg of colorant/L of culture after 3 days; the phycoerythrin level in the colorant is about 15%. A higher concentration of phycoerythrin, up to 30%, can be achieved under optimal algal culture conditions. The pinkish-red color can be used to color confections, gelatin deserts, and dairy products. The quantity of color required for 1 kg of food varies from 50 to 100 mg/kg (Yaron and Arad, 1993).

The color is stable at 60°C for 30 min and has a long shelf life at pH 6–7. As an ingredient in dry food preparations stored under low humidity conditions, it is very stable. A number of patents have been granted for use of the red color from *Porphyridium* in foods.

In addition to its coloring properties, red phycoerythrin possesses a yellow fluorescence. Opportunities for exploiting this property for special effects in food are under study. A range of foods that fluoresce under natural and UV light were prepared and tested. These include transparent lollipops made from sugar solutions, dry sugar-drop candies for cake decoration (that fluoresce under UV light), and soft drinks and alcoholic beverages that fluoresce at pH 5–6. Fluorescent color has also been added to alcoholic beverages containing up to 30% alcohol, but the shelf life for such products is short (Yaron and Arad, 1993).

This red color has not yet been approved for use in food or cosmetics. However, studies on rats fed with the dried biomass have not shown any adverse growth or histological effects. Future efforts should thus be devoted toward obtaining official approval of the color in foodstuffs for human consumption.

12.6 Phycocyanin, the Marine Blue from *Porphyridium*

A source of blue color is the red microalga *Porphyridium aerugineum*. This species is different from other red microalgae in that it lacks red phycoerythrin and its phycocyanin is C-phycocyanin rather than the R-phycocyanin

that accompanies phycoerythrin found in many red algae and in other *Porphyridium* species. However, the biochemicals produced by *Po. aerugineum* are similar to those of other red microalgae, for example, sulfated polysaccharides, carotenoids, and lipids. Alternative source of C-phycocyanin is *Spirulina platensis*.

The algal extract of *Po. aerugineum* is blue, with maximum absorbance at a wavelength of 620 nm and a red fluorescence with maximum emission at 642 nm. The main phycobiliprotein, C-phycocyanin, is the same type of phycocyanin found in most Cyanobacteria. The chromophores are composed of phycocyanobilins, conjugated to an apoprotein via thioether bonds.

Po. aerugineum is a unicellular alga, cultured under artificial or solar light in a fresh water medium supplied with CO_2, in outdoor bioreactors. Algal growth was optimized for yield and for the properties of the blue color produced. The parameters that require close monitoring are light intensity and temperature, in order to avoid stress conditions. Stress conditions result in decreased color yield and solubility and increased biosynthesis of the polysaccharides (which encapsulate the cells and are excreted into the medium). Production of the color involves centrifugal separation of the biomass, cell breakage, and extraction. Use of a salt solution rather than water as an extraction medium increases stability of the color during extraction (Yaron and Arad, 1993).

Methods for partial exclusion of the polysaccharide from the color extract in order to enhance resolubilization of the dried color were developed. These processes included either microfiltration or coprecipitation of the polysaccharide with an added positively charged polysaccharide. Microfiltration was also used to sterilize the solution containing the produced color prior to drying. Drying was performed either by lyophilization or by spray drying. After 4 days, the yield of color reached 100 mg of product/L of batch cultured for 4 days and contained 60% phycocyanin.

The blue color reached phycocyanin levels of up to 60% of the dry matter without any further separation steps. The quantity required for coloring food was 140–180 mg of color/kg of blue food or drink. The polysaccharides accompanying the product stabilize the color and contribute added value by virtue of their functional nutritional properties. If the polysaccharides are separated out, antioxidants can be added to stabilize the color (Moreira et al., 2012). The shade of the blue color produced from *Po. aerugineum* does not change with pH. The color is stable under light but sensitive to heat. Within a pH range from 4 to 5, the blue color produced from *Po. aerugineum* is stable at 60°C for 40 min (this is not typical of blue colors from Cyanobacteria). This property is important for food uses, since many food items are acidic, particularly drinks and confections. The blue color was added to clear Pepsi® (without heat application) and to Bacardi Breezer®, and these beverages did not lose their color for at least 1 month at room temperature.

The color was very stable in dry preparations. Sugar flowers for cake decoration maintained their color for years of storage. Foods prepared with the color

include gelatin and ice cream. The color was mixed with other colorants to obtain a range of shades and hues.

The blue color from *Po. aerugineum* has not been cleared for food use by the authorities, and it is not yet produced commercially. Toxicological studies carried out with other species of red microalgae have not revealed any adverse effects. Efforts should now be devoted to carrying out the required studies and procedures that will allow the use of the blue color as a substitute for synthetic colors (Eriksen, 2008).

12.7 Current Carotenoid Production Using Microorganisms

Commercial processes are already in operation or under development for the production of carotenoids by molds, yeasts, and bacteria. The production of β-carotene by microorganisms, as well as by chemical synthesis or from plant extracts, is well developed (Table 12.1), and several other carotenoids, notably lycopene, astaxanthin, zeaxanthin, and canthaxanthin, are also of interest.

12.7.1 β-Carotene

12.7.1.1 β-Carotene from B. trispora

The source organism, *B. trispora,* is a commensal mold associated with tropical plants. The fungus exists in (+) and (−) mating types (Breitenbach et al., 2012), of which the (+) type synthesizes trisporic acid, a metabolite of β-carotene. On mating the two types in a specific ratio, the (−) is stimulated by trisporic acid to synthesize large amounts of β-carotene. The mold has

TABLE 12.1

Commercial β-Carotene and β-Carotene-Containing Preparations from Various Sources

Trademark	Company	Origin
AL CARC 9004	Diana Naturals	Carrot
Altratene	Allied Industrial Corp.	Chemical synthesis
Betanat	Vitatene (Spain, DSM group)	*B. trispora*
Betatene	Cognis Nutrition & Health	*Dunaliella salina*
CaroPure	DSM	Chemical synthesis
CaroPure	DSM	*B. trispora*
Caroxan	Pot au Pin	Carrot
Lucarotin	BASF	Chemical synthesis
Mixed carotenoids	Global Palm Products	Palm oil *(Elaeis guineensis)*
Vitan	Vita-Market (Ukraine)	*B. trispora*

Data collected at Food Ingredients Europe, Paris, 2011.

shown no pathogenicity or toxicity in many experiments such as (1) standard pathogenicity tests in mice, (2) analyses of extracts of several fermentation mashes for fungal toxins, and (3) enzyme immunoassays of the final product, the β-carotene crystals, for four mycotoxins.

The production process proceeds essentially in two stages. Glucose and corn steep liquor can be used as carbon and nitrogen sources. Whey, a by-product of cheese manufacture, has also been considered, with strains adapted to metabolize lactose. In the initial fermentation process, seed cultures are produced from the original strain cultures and subsequently used in an aerobic submerged batch fermentation to produce a biomass rich in β-carotene (Papaioannou and Liakopoulou-Kyriakides, 2010). In the second stage, the recovery process, the biomass is isolated and transformed into a form suitable for isolating the β-carotene, which is extracted with ethyl acetate, suitably purified and concentrated, and the β-carotene crystallized. The final product is either used as crystalline β-carotene (purity >96%) or formulated as a 30% suspension of micronized crystals in vegetable oil. The production process is subject to Good Manufacturing Practices (GMP) procedures and adequate control of hygiene and raw materials. The biomass and the final crystalline product comply with an adequate chemical and microbiological specification, and the final crystalline product also complies with the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and EU specifications as set out in Directive 95/45/EC for coloring materials in food.

The first β-carotene product from *B. trispora* was launched in 1995 at the Food Ingredients Europe business meeting in London. Following the optimization of the fermentation process, many aspects had to be addressed before the product could be marketed:

- *The microorganism*. A fungus isolated from a natural environment, not genetically modified; yield improvement achieved by classical genetics
- *Guidelines for labeling*. Natural β-carotene; natural β-carotene from *B. trispora*; fermentative, natural β-carotene; natural β-carotene from a fermentative source
- *Lobbying from other β-carotene producers (nature-identical, mixed carotenes from palm oil, β-carotene from the microalgae Dunaliella)*. The EU Health and Consumer Protection Directorate General was asked to give an opinion on the safety of β-carotene from a dried biomass source, obtained from a fermentation process with *B. trispora*, for use as a coloring matter for foodstuffs
- *Safety of the fermentation-produced β-carotene*. High-pressure liquid chromatography (HPLC) analysis, stability tests, and microbiological tests have shown that the β-carotene obtained by co-fermentation of *B. trispora* complies with the EC specification for E 160 aii, also including the proportions of *cis* and *trans* isomers, and is free of mycotoxins or other toxic metabolites. Tests in vitro for gene mutations and chromosomal

TABLE 12.2

Isomers Described in "β-Carotene" from Various Sources

	Carotenoids (%)			
Source	All *Trans*-β-Carotene	*cis*-β-Carotene	α-Carotene	Other
Fungus (*B. trispora*)	94	3.5	0	2.5
Chemical synthesis	98	2	0	0
Algae (*Du. salina*)	67.4	32.6	0	0
Palm oil	34	27	30	9

aberrations with the β-carotene produced by the manufacturer in the EU showed it to be free of genotoxic activity. In a 28-day feeding study in rats with the β-carotene manufactured in the EU, no adverse findings were noted at a dose of 5% in the diet, the highest dose level used. In conclusion, evaluation of the source organism and the production process yielded no grounds to suppose that the final crystalline product, β-carotene, differs from the chemically synthesized β-carotene used as a food colorant. The final crystalline fermentation product has been shown to comply with the specification for β-carotene E 160 aii listed in Directive 95/45/EC. The Committee considers that "β-carotene produced by co-fermentation of *Blakeslea trispora* is equivalent to the chemically synthesized material used as food colorant and is therefore acceptable for use as a colouring agent for foodstuffs" (Table 12.2).

Today there are other industrial productions of β-carotene from *B. trispora* in Russia and Ukraine and in León (Spain). The process has been developed to yield up to 30 mg of β-carotene/g dry mass or about 3 g/L. *B. trispora* is now also used for the production of lycopene (ACNFP, 2004).

12.7.1.2 β-Carotene from Phycomyces blakesleeanus

Another mold, *Ph. blakesleeanus*, is a potential source for various chemicals including β-carotene. The carotene content of the wild type grown under standard conditions is modest, about 0.05 mg/g dry mass, but some mutants accumulate up to 10 mg/g. As for *B. trispora*, sexual stimulation of carotene biosynthesis is essential and can increase yields up to 35 mg/g. The most productive strains of *Phycomyces* achieve their full carotenogenic potential on solid substrates or in liquid media without agitation. *B. trispora* is more appropriate for production in usual fermentors.

12.7.1.3 β-Carotene from Mucor circinelloides

Mu. circinelloides wild type is yellow because it accumulates β-carotene as the main carotenoid. The basic features of carotenoid biosynthesis, including photoinduction, are similar in *Phycomyces* and *Mucor*. *Mu. circinelloides*

responds to blue light by activating biosynthesis. Wild-type strains grown in darkness contain minimal amounts of β-carotene because of the low levels of transcription of the structural genes for carotenogenesis (Almeida and Cerda-Olmedo, 2008). When exposed to a light pulse, the level of transcription of these genes increases strongly, leading to high pigment concentrations. *Mu. circinelloides* is a dimorphic fungus that grows either as yeast cells or in a mycelium form, and research is now focused on yeastlike mutants that could be useful in a biotechnological production.

12.7.2 Lycopene

Lycopene is an intermediate in the biosynthesis of all dicyclic carotenoids, including β-carotene. In principle, therefore, blocking the cyclization reaction and the cyclase enzyme by mutation or inhibition will lead to the accumulation of lycopene. This strategy is employed for the commercial production of lycopene.

12.7.2.1 Lycopene from B. trispora

A process for lycopene production by *B. trispora* is now established, with the aim of marketing this product in Europe for use as a nutritional food ingredient and dietary supplement or a food color. Lycopene is an intermediate in the β-carotene biosynthetic pathway and microbial strains that accumulate lycopene are easy to obtain by mutagenesis, molecular biology, or use of inhibitors. In the commercial process, imidazole or pyridine is added to the culture broth to inhibit the enzyme lycopene cyclase. The product, predominantly (all-*trans*)-lycopene, is formulated into a 20% or 5% suspension in sunflower oil with α-tocopherol at 1% of the lycopene level. Also available is an α-tocopherol-containing 10% and 20% lycopene cold water dispersible product. Lycopene oil suspension is intended for use as a food ingredient and in dietary supplements. The proposed level of use for lycopene in food supplements is 20 mg/day (ACNFP, 2004).

Approval for the use of lycopene from *B. trispora* was sought under regulation (EC) No 258/97 of the European Parliament and the Council concerning novel foods and novel food ingredients. The European Food Safety Authority was also asked to evaluate this product for use as a food color. It was stated in the application that it is likely that lycopene from *B. trispora* in food supplements would simply replace those supplements containing lycopene from other sources that are already being marketed so that overall consumption levels would not increase. However, incorporation of lycopene into foods would result in additional intake.

The dossier first satisfied the United Kingdom Advisory Committee on Novel Foods and Processes (ACNFP) in 2004 (ACNFP, 2004), but the Panel from the European Food Safety Authority, in opinions adopted on April 21 and October 5, 2005, was unable to conclude whether the proposed use levels of lycopene from *B. trispora* would be safe. In 2012, the placing on the market of lycopene from *B. trispora* as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament is fully authorized.

Existing authorizations and evaluations on lycopene from various sources are quite numerous. Lycopene, extracted from tomatoes, is authorized as food coloring agent within the EU (E160d) (Directive 94/36/EC) and the United States (CDR 21 73.295). This restricts the amount that can be added to foods. Lycopene was evaluated by the Scientific Committee on Food in 1975 when it was unable to allocate an Acceptable Daily Intake (ADI) but felt able to accept the use of lycopene prepared from natural foods by physical processes, without further investigations, as a coloring matter in food, provided that the amount consumed does not differ significantly from the amount consumed through the relevant foodstuffs. This opinion was reiterated by SCF in 1989. When JECFA evaluated lycopene from natural sources in 1977, they postponed a decision because of lack of data. In 1999, the SCF evaluated synthetic lycopene, but the available data were not sufficient to allow for an acceptance. The SCF concluded: "The Committee is not able to allocate an ADI and considers its use in food is unacceptable at present." Synthetic lycopene is currently used as food ingredient but is not approved for coloring matters within the EU, and it is considered Generally Recognized as Safe (GRAS) in the United States (GRAS notice No GRN 000119). In Australia and New Zealand, lycopene is permitted for use as a food color in processed foods in accordance with GMP under Schedule 3 of Standard 1.3.1 in the Food Standards Code. In Japan, tomato color, defined as "a substance composed mainly of lycopene obtained from tomato fruits," is permitted for use as a food additive under the Food Sanitation Law.

To summarize, the lycopene from *B. trispora* is considered by the EFSA Panel to be nutritionally equivalent to natural dietary lycopene, but further safety trials are necessary. While the toxicity data on lycopene from *B. trispora* and on lycopene from tomatoes do not give indications for concern, nevertheless, these data are limited and do not allow an ADI to be established. The main concern is that the proposed use levels of lycopene from *B. trispora* as a food ingredient may result in a substantial increase in the daily intake of lycopene compared to the intakes solely from natural dietary sources (Vitatene™, personal communication).

12.7.2.2 Lycopene from Fusarium sporotrichioides

The fungus *F. sporotrichioides* has been genetically modified to manufacture lycopene from the cheap corn fiber material, the "leftovers" of making ethanol. Corn fiber is abundant (the U.S. ethanol industry generates 4 million tons annually) and costs about 5 cents a pound. Distiller's dry grains with solubles could also be used as a substrate. Using a novel, general method for the sequential, directional cloning of multiple DNA sequences, the isoprenoid pathway of the fungus was redirected toward the synthesis of carotenoids. Strong promoter and terminator sequences from the fungus were added to carotenoid biosynthetic genes from the bacterium *Erwinia uredovora*, and the chimeric genes were assembled, introduced in the fungus, and expressed

at levels comparable to those observed for endogenous biosynthetic genes. Cultures in laboratory flasks produced 0.5 mg lycopene/g dry mass within 6 days, and this is predicted to increase in the next few years.

12.7.3 Astaxanthin

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is widely distributed in nature and is the principal pigment in crustaceans and salmonid fish. The carotenoid imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the market place. Since animals cannot synthesize carotenoids, the pigments must be supplemented in the feeds of farmed species. Salmon and trout farming is now a huge business and feeding studies have shown that astaxanthin is very effective as a flesh pigmenter. There are also reports of beneficial actions of astaxanthin for human health, so its use in supplements is of interest (Seabra and Pedrosa, 2010).

12.7.3.1 Astaxanthin from X. dendrorhous*, Formerly* Phaffia rhodozyma

Among the few astaxanthin-producing microorganisms, *X. dendrorhous* is one of the best candidates for commercial production of (3*R*, 3′*R*)-astaxanthin. Many academic laboratories and several companies have developed processes suitable for industrial production (Rodriguez-Saiz et al., 2010).

Several reports have shown that constituents in the medium, among other environmental factors, affect astaxanthin production in this yeast. The effects of different nutrients on *X. dendrorhous* have generally been studied in media containing complex sources of nutrients such as peptone, malt, and yeast extracts. By-products from agriculture were also tested, such as molasses, enzymatic wood hydrolysates, corn wet-milling coproducts, bagasse or raw sugarcane juice, date juice, and grape juice. Although such media are often convenient because they contain all nutrients, they suffer from the disadvantage of being undefined and sometimes variable in composition; this may mask important nutritional effects. For this reason, several studies have yielded results that are difficult to interpret in detail because of inadequate characterization of growth-limiting factors in the media. Thus, in order to elucidate the nature of nutritional effects as far as possible, chemically defined or synthetic media were used by some authors. In one study, 11 strains were assayed for their ability to utilize 99 different compounds as single carbon source. In a second study, carotenoid biosynthesis was increased at low ammonium or phosphate levels and stimulated by citrate. Factorial design and response surface methodology could be used to optimize the astaxanthin production. The optimal conditions stimulating the highest astaxanthin were 19.7°C temperature, 11.25 g/L carbon concentration, 6.0 pH, 5% inoculum, and 0.5 g/L nitrogen concentration. Under these conditions, the astaxanthin content was 8.1 mg/L. Fermentation strategy also has an impact on growth and carotenoid production of *X. dendrorhous*,

as shown with fed-batch (e.g., limiting substrate is fed without diluting the culture) or pH-stat (i.e., a system in which the feed is provided depending on the pH) cultures. The highest biomass obtained was 17.4 g/L.

A major drawback in the use of *X. dendrorhous* is that disruption of the cell wall of yeast biomass is required before addition to animal diet, to allow intestinal absorption of the pigment. Several chemical, physical, autolytic, and enzymatic methods for cell wall disruption have been described, including a two-stage batch fermentation technique. The first stage was for "red yeast" cultivation. The second stage was the mixed fermentation of the yeast and *Bacillus circulans*, a bacterium with a high cell-wall-lytic activity.

Another starting point in optimization experiments is the generation of mutants, but metabolic engineering of the astaxanthin biosynthetic pathway is now attractive. It should be possible to manage carbon fluxes within the cell and resolve competition between enzymes such as phytoene desaturase and lycopene cyclase.

The case of *X. dendrorhous* (*Pha. rhodozyma*) is peculiar as hundreds of scientific papers and patents deal with astaxanthin production by this yeast, but the process has not been economically efficient up to now (Schmidt et al., 2011). New patents are filed almost each year, with improvement in astaxanthin yield, for example, 3 mg/g dry matter in a U.S. Patent.

12.7.3.2 Astaxanthin from Agrobacterium aurantiacum *and Other Bacteria*

Compared to the huge research effort devoted to *X. dendrorhous*, astaxanthin production by *Ag. aurantiacum* has been investigated to a lesser extent. The first description of astaxanthin biosynthesis in this bacterium was published in 1994. Astaxanthin is 1 of 10 carotenoids present. The biosynthetic pathway, the influence of growth conditions on carotenoid production, and the occurrence of astaxanthin glucoside were described in two subsequent papers, but commercial processes have not yet been developed (Yokoyama et al., 1995).

Numerous screenings have been conducted with the aim of characterizing new biological sources of astaxanthin, and positive targets were isolated such as *Paracoccus carotinifaciens* or *Halobacterium salinarum*. The latter is particularly interesting because (1) the extreme NaCl concentrations (about 20%) used in the growth medium prevent contamination with other organisms so no particular care has to be taken with sterilization; (2) NaCl concentrations under 15% induce bacterial lysis, so that no special cell breakage technique is necessary; and (3) pigments may be extracted directly with sunflower oil instead of organic solvents. This eliminates possible toxicity problems due to trace amounts of acetone or hexane and facilitates pigment assimilation by animals.

12.7.4 Zeaxanthin

Zeaxanthin (-β,β-carotene-3,3′-diol) can be used, for example, as an additive in feeds for poultry to intensify the yellow color of the skin or to accentuate the

color of the yolk of their eggs (Breithaupt, 2007). It is also suitable for use as a colorant, for example, in the cosmetics and food industries, and as a health supplement that may help to prevent age-related macular degeneration.

In the mid-1960s, several marine bacteria were isolated that produced zeaxanthin. Cultures of a *Flavobacterium* sp. (ATCC 21588, classified under the accepted taxonomic standards of that time) in a defined nutrient medium containing glucose or sucrose, as carbon source, were able to produce up to 190 mg of zeaxanthin/L, with a concentration of 16 mg/g dried cellular mass. One species, *Flavobacterium multivorum* (ATCC 55238), is currently under investigation in many studies (Bhosale et al., 2003).

Recently, a zeaxanthin-producing "*Flavobacterium*" was reclassified as a new *Paracoccus* species, *Par. zeaxanthinifaciens*, and earlier findings that IPP biosynthesis occurs exclusively *via* the mevalonate pathway were confirmed. A second strain, isolated in a mat from an island of French Polynesia, is peculiar as it also produces exopolysaccharides.

Sphingobacterium multivorum, the new name for *Fl. multivorum*, was recently shown to utilize the alternative deoxyxylulose phosphate (DXP) pathway. A strain was constructed for overproduction of zeaxanthin in industrial quantities.

As more bacteria are examined, the distribution of the mevalonate and DXP pathways will become better defined, thus facilitating the metabolic engineering of microorganisms with improved production of commercially important isoprenoid compounds including carotenoids.

12.7.5 Canthaxanthin

Canthaxanthin (-β,β-carotene-4,4′-dione) has been used in aquafeed for many years in order to impart the desired flesh color in farmed salmonids. A *Bradyrhizobium* sp. strain was described as a canthaxanthin producer and the carotenoid gene cluster was fully sequenced. Interest in canthaxanthin is decreasing since the discovery of extreme overdosage, i.e., the deposition of minute crystals in the eye, a fact leading to adverse media attention in the past, and some pressure to limit its use in aquafeeds (Chandi and Gill, 2011).

A second bacterium under scrutiny for canthaxanthin production is *Haloferax alexandrinus*, which belongs to the extremely halophilic Archaea, chemo-organotrophic organisms that satisfy some of their energy requirements with light. Members of the family *Halobacteriaceae* are characterized by red-colored cells, the color in most cases being due to the presence of C_{50}-carotenoids (especially bacterioruberins) as the major carotenoids. Some species have been reported to produce C_{40}-carotenoids and ketocarotenoids as minor carotenoids. Recently, the biotechnological potential of these members of the Archaea has increased because of their unique features, which facilitate many industrial procedures. For example, no sterilization is required, because of the extremely high NaCl concentration used in the growth medium (contamination by other organisms is avoided). In addition, no cell-disrupting devices are required, as cells lyse spontaneously in fresh water.

A 1 L scale cultivation of the cells in flask cultures (6 days) under nonaseptic conditions produced 3 g dry mass, containing 6 mg total carotenoid and 2 mg canthaxanthin. Further experiments in a batch fermentor also demonstrated increases in the biomass concentration and carotenoid production.

A third example is *Gordonia jacobea* (CECT 5282), a Gram-positive, catalase-negative G+C 61% bacterium, which was isolated in routine air sampling during screening for microorganisms that produce pink colonies. Analysis of the carotenoid extracts by HPLC-MS revealed that the main pigment in the isolates is canthaxanthin. The low carotenoid content (0.2 mg/g dry mass) in the isolate does not support an industrial application, but after several rounds of mutations, a hyperpigmented mutant (MV-26) was isolated, which accumulated six times more canthaxanthin than the wild-type strain. Apart from their high pigment production, the advantages of mutants of this species from the industrial point of view are (1) the optimal temperature for growth and carotenogenesis 30°C, which is usual in fermentors; (2) the use of glucose, an inexpensive carbon source, for optimal growth and pigmentation; and (3) the fact that >90% of the total pigments can be extracted directly with ethanol, a nontoxic solvent allowed for human and animal feed. Many other culture media were tested, giving canthaxanthin from 1 to 13.4 mg/L.

12.7.6 Torulene and Torularhodin

Yeasts in the genus *Rhodotorula* synthesize carotenoids, mainly torulene (3′,4′-didehydro-β,ψ-carotene) and torularhodin (3′,4′-didehydro-β,ψ-caroten-16-oic acid) accompanied by very small amounts of β-carotene. Most of the research has focused on the species *Rhodotorula glutinis*, but some papers deal with other species such as *R. gracilis*, *R. rubra*, and *R. graminis* (Frengova and Beshkova, 2009).

Feed supplemented with a *Rhodotorula* cell mass has been found to be safe and nontoxic in animals. Its use in the nutrition of laying hens has also been documented. As the β-carotene content in wild strains of *R. glutinis* is low, efforts have been made to increase it through strain improvement, mutation, medium optimization, and manipulation of culture conditions (temperature, pH, aeration, C/N ratio). These studies mainly resulted in an increased yield of torulene and torularhodin, which are of minor interest, though some did succeed in increasing the β-carotene content up to about 70 mg/L.

12.8 Summary and Future Prospects

There are several advantages of microorganisms for the study of biosynthesis and function of pigments. Bacteria and fungi offer a tremendous resource in that they produce hundreds to thousands of various pigmented molecules.

It is likely that many more natural pigments will be isolated from this biomass (Dufossé et al., 2005).

Synthetic pigments traditionally used by food or cosmetic processors continue to be utilized with success; however, with the increasing consumer preference for natural food additives, natural colorants from plants is now a big business and most of the research efforts within the scientific field of colorants are conducted on natural ones. Regarding bacteria, yeast, or fungi (Table 12.3), despite common belief about the high production cost of fermentation pigments, two initiatives started in Europe these last years: β-carotene from the filamentous

TABLE 12.3

Microbial Production of Pigments (Already in Use as Natural Colorants or with High Potential in This Field)

Molecule	Color	Microorganism	Status[a]
Ankaflavin	Yellow	*Monascus* sp. (fungus)	IP
Anthraquinone	Red	*P. oxalicum* (fungus)	IP
Astaxanthin	Pink-red	*X. dendrorhous* (yeast), formerly *Pha. rhodozyma*	DS
Astaxanthin	Pink-red	*Ag. aurantiacum* (bacteria)	RP
Astaxanthin	Pink-red	*Par. carotinifaciens* (bacteria)	RP
Canthaxanthin	Dark red	*Bradyrhizobium* sp. (bacteria)	RP
Canthaxanthin	Dark red	*H. alexandrinus* (bacteria)	RP
Canthaxanthin	Dark red	*G. jacobea* (bacteria)	DS
Lycopene	Red	*B. trispora* (fungus)	DS
Lycopene	Red	*F. sporotrichioides* (fungus)	RP
Melanin	Black	*Saccharomyces neoformans* var. *nigricans* (yeast)	RP
Monascorubramin	Red	*Monascus* sp. (fungus)	IP
Naphthoquinone	Deep bloodred	*Cordyceps unilateralis* (fungus)	RP
Riboflavin	Yellow	*A. gossypii* (fungus)	IP
Rubrolone	Red	*Streptomyces echinoruber* (bacteria)	DS
Rubropunctatin	Orange	*Monascus* sp. (fungus)	IP
Torularhodin	Orange-red	*Rhodotorula* sp. (yeast)	DS
Zeaxanthin	Yellow	*Flavobacterium* sp. (bacteria)	DS
Zeaxanthin	Yellow	*Par. zeaxanthinifaciens* (bacteria)	RP
Zeaxanthin	Yellow	*S. multivorum* (bacteria)	RP
β-carotene	Yellow-orange	*B. trispora* (fungus)	IP
β-carotene	Yellow-orange	*F. sporotrichioides* (fungus)	RP
β-carotene	Yellow-orange	*Mu. circinelloides* (fungus)	DS
β-carotene	Yellow-orange	*Neurospora crassa* (fungus)	RP
β-carotene	Yellow-orange	*Ph. blakesleeanus* (fungus)	RP
Unknown	Red	*Penicillium purpurogenum* (fungus)	DS
Unknown	Red	*Pa. sinclairii* (fungus)	RP

[a] IP, Industrial production; DS, development stage; RP, research project.

fungi, *B. trispora* (produced by Gist-Brocades now DSM; approved in 2000 by the EU Scientific Committee on Food Safety) and arpink red from *P. oxalicum* (manufactured by Ascolor). These companies invested a lot of money as any combination of new source and/or new pigment drives a lot of experimental work, process optimization, toxicological studies, regulatory issues, and tremendous paper work. Another development under process is the production of lycopene using *B. trispora* by Vitatene, a subsidiary of Spanish penicillin firm Antibioticos (now a subsidiary of DSM, 2011). Exploration of fungal biodiversity is still going on, with special interest in water-soluble pigments. The case of *X. dendrorhous* (*Pha. rhodozyma*) is very peculiar as hundreds of scientific papers and patents deal with astaxanthin production using this yeast, and the process has not been economically efficient up to now. Microorganisms could either be used for the biosynthesis of "niche" pigments not found in plants, such as aryl carotenoids. Carotenoids play an exceptional role in the fast-growing "over-the-counter medicine" and "nutraceutical" sector. Among carotenoids under investigation for coloring or for biological properties, a small number are available from natural extracts or chemical synthesis. The list is rather short compared to the long list of 700 entries in the *Carotenoid Handbook* (Britton et al., 2004). With imagination, biotechnology could be a solution for providing additional pigments including interesting aryl carotenoids. Isorenieratene (Φ,Φ-carotene) and its monohydroxy and dihydroxy derivatives can be produced by bacteria, i.e., *Brevibacterium linens*, *Streptomyces mediolani*, or *Mycobacterium aurum*.

Research projects mixing molecular biology and pigments were investigated all over the world and it seems that current productions are not effective in terms of final yield. Nowadays, combinatorial genetic engineering is being addressed, based on an increasing number of known carotenogenic gene sequences. By combining genes, some authors were able to obtain more efficient biosynthesis, or new carotenoids, never described in nature, such as multi-hydroxylated ones, which could be very efficient as antioxidants.

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Article 2

☒ Filamentous fungi are large-scale producers of pigments and colorants for the food industry. Dufossé L., Fouillaud M., Caro Y., Mapari S.A.S., Sutthiwong N.. Current Opinion in Biotechnology, 2014, 26, 56-61.

Lié à la mondialisation des tendances de la recherche, au style de vie plus sain, et à la croissance du marché pour les colorants alimentaires naturels dans les pays économiquement à croissance rapide dans le monde entier, des champignons filamenteux sont à l'étude comme des sources facilement disponibles de diverses familles de colorants. La pénétration des ingrédients dérivés de la fermentation dans les industries alimentaires est en augmentation année après année. Des exemples pourraient être pris dans les domaines suivants: les agents épaississants ou gélifiants, les composés aromatiques, les acidulants, etc. Des efforts ont été faits afin de réduire le coût de la production de pigments produits par la fermentation microbienne par rapport à celles des pigments synthétiques, ou des pigments extraits de sources naturelles. La commercialisation réussie de pigments naturels provenant de microalgues (les sources non conventionnelles) ou extraits de plantes à fleurs (la source conventionnelle), en tant que colorants alimentaires et suppléments nutritionnels, reflète la présence d'un marché de niche dans lequel les consommateurs sont prêts à payer une prime pour des "ingrédients naturels et sains". Parmi les autres sources non conventionnelles, des champignons filamenteux sont connus pour produire une gamme extraordinaire de pigments qui comprennent plusieurs classes chimiques.

Ainsi, le présent article souligne le rôle crucial joué par les champignons filamenteux, rôle qu'ils sont susceptibles de continuer à jouer à l'avenir, en tant qu'usines de cellules microbiennes pour la production de pigments de qualité alimentaire en raison de la polyvalence de leur couleur de pigments et le profil chimique, la facilité de la culture à grande échelle, et l'histoire à long terme des souches productrices étudiées.

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Current Opinion in Biotechnology

Filamentous fungi are large-scale producers of pigments and colorants for the food industry

Laurent Dufossé[1], Mireille Fouillaud[1], Yanis Caro[1], Sameer AS Mapari[2,4] and Nuthathai Sutthiwong[1,3]

With globalization in the research trends, healthier life styles, and the growing market for the natural food colorants in the economically fast-growing countries all over the world, filamentous fungi are being investigated as readily available sources of chemically diverse colorants. With two selected examples, polyketide-*Monascus*-like pigments from the new fungal production strains, and the promising and yet unexplored hydroxy-anthraquinoid colorants, the present review highlights exciting recent findings, which may pave the way for alternative and/or additional biotechnological processes for the industrial production of natural food colorants of improved functionality. As an additional aspect, marine fungi are discussed as potential sources of novel pigments of numerous color hues and atypical chemical structures.

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Introduction

Penetration of the fermentation-derived ingredients into the food industries is increasing year after year. Examples could be taken from the following fields: thickening or gelling agents (xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium glutamate), flavor compounds (gamma-decalactone, diacetyl, methyl-ketones), acidulants (lactic acid, citric acid), etc. Efforts have been made in order to reduce the production costs of pigments produced by microbial fermentation compared to those of synthetic pigments or pigments extracted from natural sources. The successful marketing of natural pigments derived from algae (non-conventional sources) or extracted from flowering plants (conventional sources), both as food colorants and nutritional supplements, reflects the presence, and importance of niche markets in which consumers are willing to pay a premium for 'natural healthy ingredients'. Among non-conventional sources, filamentous fungi are known to produce an extraordinary range of pigments that include several chemical classes such as carotenoids, melanins, flavins, phenazines, quinones, and more specifically monascins, violacein or indigo. The success of any class of pigment produced by fermentation depends upon its acceptability by the consumers, regulatory approval, and the capital investment required to bring the product to the market. Twenty years ago, personnel from food industries expressed doubts about the successful commercialization of fermentation-derived food grade pigments because of the high capital investment requirements for fermentation facilities, and the extensive and lengthy toxicity studies required by the regulatory agencies. Public perception of fungal-derived products for food use also had to be taken into account. Now-a-days some fermentative food grade pigments from filamentous fungi are existing in the market: *Monascus* pigments, Arpink red™ from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, lycopene and β-carotene from *Blakeslea trispora*. The production yield in the case of β-carotene as an example, could be as high as 17 g/L of the *B. trispora* culture medium [1].

Thus, the present article emphasizes the crucial role that filamentous fungi are currently playing and are likely to continue to play in the future as microbial cell factories for the production of food grade pigments because of the versatility in their pigment color and chemical profile, amenability for easy large scale cultivation, and a long-term history of well-studied production strains. In this review, we draw attention of both the academia and the food industry to some stimulating research findings in the area of fungal pigments. We illustrate this by polyketide-*Monascus*-like pigments from the new non-mycotoxigenic, and thus potentially safe fungal production strains, and the promising and yet unexplored hydroxy-anthraquinoid pigments with an amenability to be cultivated for the large scale industrial production as natural food colorants of microbial origin. Additionally, fungi from marine

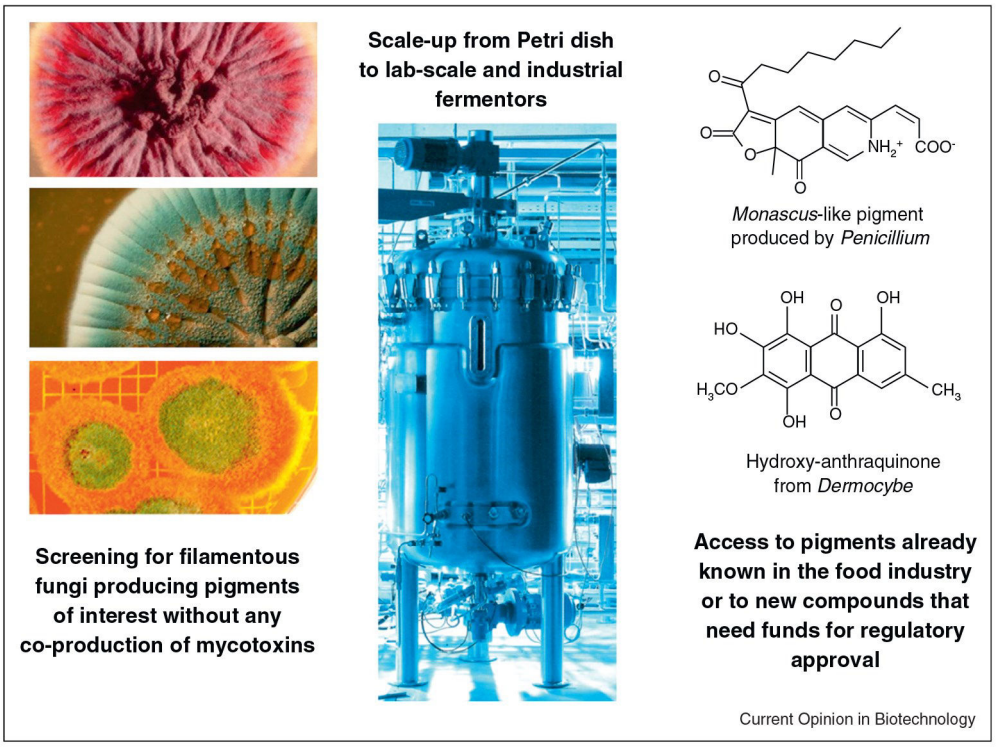
ecological niches are discussed as potential producers of novel color hues and structures, and the ways to explore the metabolic potential of these pigment producing marine fungi.

New non-mycotoxigenic fungal workhorses for the production of polyketide pigments as food colorants

The red and the yellow polyketide pigments — from the well-known fungus *Monascus* sp. have been commercially produced and legally used as food colorants in the form of pigment extracts and/or as traditionally used dried fermented red rice powder in the South East Asia for more than thousand years [2]. *Monascus* pigments are not approved as food colorants both in the European Union (EU) and the United States (US), mainly due to the risk of the possible contamination by the nephrotoxic, and hepatotoxic metabolite citrinin. There have been controversial views over the safety of these pigments though co-production of a toxic metabolite together with the main target compound by a fungal host is not alien and one of the examples is the case of a mycelial fungal food product Quorn^{TM} by *Fusarium venenatum* whereby a potent cytotoxic metabolite 4,15-diacetoxyscirpenol is produced. Another example is the production of fumonisins by one of the well-known industrial workhorses, *Aspergillus niger*. The point is that the fungal producer is designated GRAS (Generally Recognized As Safe) for the processes involved for the food use and not the organism as such. This implies that the safe production of *Monascus* pigments can be designed keeping the hazard analysis and critical control points in mind. Nonetheless, the presence of the mycotoxin citrinin issue has triggered investigations to find possibilities for minimizing citrinin accumulation by firstly, manipulating culturing conditions [3]; secondly, developing strains incapable of synthesizing citrinin by metabolic engineering [4], and finally, simply screening for genera other than *Monascus* that produce polyketide pigments [5] (Figure 1).

Some strains among *Talaromyces* species (formerly *Penicillium* sp.) viz. *Talaromyces aculeatus*, *T. funiculosus*, *T. pinophilus*, and *T. purpurogenus* have been discovered to produce *Monascus*-like polyketide azaphilone (MPA) pigments without co-producing citrinin or any other known mycotoxins using chemotaxonomic rationale [6,7]. *Epicoccum nigrum* is also shown to be non-mycotoxigenic fungal producer [7] of polyketide pigment orevactaene with antioxidant property [8]. The red and the yellow pigment extracts from *T. aculeatus* and *E. nigrum*, respectively have been shown to exhibit enhanced photostability over the commercially available red *Monascus* colorant and

Figure 1



Using the filamentous fungi as microbial cell factories for the production of food pigments (description in three steps, from left to right: Firstly, filamentous fungi are used as pigment producers — biodiversity, versatility; secondly, fermentors allow the easy scale-up of the production; and finally, two examples of pigments produced).

turmeric in liquid food model systems [9]. Recently, *T. purpurogenus* (formerly *Penicillium purpurogenum*) and related species, such as *Talaromyces amestolkiae*, *Talaromyces ruber*, and *Talaromyces stollii*, have also been reported to produce MPA pigments [10]. Strains of these species are likely to be non-mycotoxigenic and non-pathogenic to humans; however, their individual mycotoxin profiles and pigment producing abilities have yet to be explored. Currently, the pigment production potential and pigment producing cellular mechanisms of strains of *T. purpurogenus* are being investigated [11–13]. More recently, a patent has been granted for a submerged cultivation method for some of the non-mycotoxigenic strains of *Talaromyces* sp. whereby, the concentration of pigments was significantly enhanced and the number of pigment constituents was significantly reduced with MPA pigment PP-V [(10Z)-12-carboxyl-monascorubramine] being the major compound [14].

Thus, recent research activities indicate that the above-mentioned new fungal cell factories have advantages in firstly, potentially eliminating the risk of contamination by mycotoxins especially citrinin; secondly, reducing the pigment constituents and thereby easier characterization and quality control; and finally, providing the colorants with improved stability. In addition, the new fungal production strains have a potential to be engineered to produce a new generation of natural functional food ingredients with bioactive properties.

Fungal hydroxyanthraquinoid (HAQN) pigments as potential food colorants

HAQN pigments are widespread in nature (plants, insects, lichens) and have also been found abundantly in microorganisms, particularly in filamentous fungi belonging to the genera *Penicillium* sp. and *Aspergillus* sp., with different color hues. For example, the pigment emodin is isolated from the strains of *Penicillium citrinum* and *P. islandicum*.

As a first commercial product within this chemical family, the natural food colorant Arpink red™ (now Natural Red™) is manufactured by the Czech company (Ascolor Biotech followed by Natural Red) and has been claimed to be produced by fermentation and bioprocess engineering using the fungal strain *Penicillium oxalicum* var. Armeniaca CCM 8242, a soil isolate.

Some strains of *Aspergillus* sp. (*A. glaucus*, *A. cristatus* and *A. repens*) were found to produce known yellow and red HAQN compounds, such as emodin (yellow), physcion (yellow), questin (yellow to orange-brown), erythroglaucin (red), catenarin (red) and rubrocristin (red) [15••]. However, using strains of *Aspergillus* and *Penicillium* sp., several known mycotoxins are co-produced in the medium, for example, secalonic acid D, oxaline, citrinin, tanzawaic acid A, cyclochlorotine, islanditoxin, luteoskyrin, erythroskyrin, rugulosin or aspergiolide A. Many of these mycotoxins are pigmented and are naphtoquinones by chemical nature. All these fungal secondary metabolites (the yellow and the red HAQN pigments that show substitution on both aromatic rings as well as the naphtoquinone-type mycotoxins) biosynthetically arise by the same polyketide pathway. This infers that these fungal strains are not safe, and therefore, cannot be used for the production strains of HAQN pigments as potential natural food grade colorants. Traditional mutagenesis and/or metabolic engineering methods to eliminate the production of mycotoxin(s) should be investigated as an alternative strategy.

Species of *Eurotium* sp. (*E. amstelodami*, *E. chevalieri* and *E. herbariorum*) have been found to produce the yellow pigment physcion and the red pigment erythroglaucin; however they have been reported to produce, in addition, the mycotoxin echinulin and two benzaldehyde coloring compounds: flavoglaucin (yellow) and auroglaucin (red) [16••]. Along similar lines, co-production of the red HAQN pigments and mycotoxins, such as fusaric acid, nectriafurone, monoliformin and gibepyrones, has been shown in strains of *Fusarium oxysporum* isolated from roots of diseased citrus trees.

Apart from those mycotoxigenic fungi, other filamentous fungi have the ability to produce known HAQN pigments which arise biosynthetically through the polyketide pathway, without co-production of mycotoxins. As an example a strain of *Dermocybe sanguinea* (=*Cortinarius sanguineus*) has been identified as a producer of the red HAQN glycoside dermocybin-1-β-D-glycopyranoside, giving the typical red color of the fruiting body and the spores, together with the pigments emodin and physcion [17].

As of now, HAQN pigments used in food are from plants (European madder root color, that is, *Rubia tinctoria*, sold in Japan up to 2004) or insects (carminic acid extracted from cochineal insects; *Dactylopius coccus*); however there is an increasing interest both from the academia and the industry about the readily available microbial sources.

Fungi from marine ecological niches as novel sources of chemically diverse pigments

Recent literature abundantly reports the interest for marine organisms with respect to the production of new molecules and, among them, new pigments. Indeed, many marine ecological niches are still unexplored and it seems plausible that unique features of marine environments such as high salinity, low temperature, lack of light, and high pressure can be the inducers of unique substances synthesized by marine microorganisms. The potential of marine microorganisms to produce unique and original molecules could therefore come from specific metabolic or genetic adaptation processes to meet very specific combinations of physico-chemical parameters. For now, the highest diversity of marine fungi seems

to be found in tropical regions, mainly in tropical mangroves which are extensively studied because of their high richness in organic matter favorable to the development of these heterotrophic microorganisms. Anyway, it seems obvious that in extreme environments the fungal species with pigmented cell walls in the spores and mycelium, can tolerate dehydration–hydration cycles and high solar radiation better than the moniliaceous fungi, whose cells are devoid of pigments. For example melanin, a black phenolic pigment which has a significant antioxidant activity and sporopollenin (brown product of oxidative polymerization of β-carotene) are very common in many fungi (dematiaceous hyphomycetes) and may protect the biological structures, giving them an excellent durability and a high potential for survival in hostile environments. Another example is the deep green pigment cycloleucomelone (terphenylquinone) from an *A. niger* strain isolated from the mediterranean sponge *Axinella damicornis*, as well as from its terrestrial counterparts [18].

Marine fungi are also able to produce bright colors, from yellow to red, mainly belonging to polyketides. Indeed, several review papers illustrate that polyketides seem to dominate marine natural products of fungal origin [19••]. As examples, yellow pigments physcion and macrosporin have been reported to be extracted from the endophytic *Alternaria* sp., isolated from the fruit of the marine mangrove tree *Aegiceras corniculatum* in Zhanjiang, Guangdong (South China Sea) [20]. The orange questin, the yellow asperflavin, and the brown 2-O-methyleurotinone have been reported to be produced by *Eurotium rubrum* from the inner tissue of the stem of the marine mangrove plant *Hibiscus tiliaceus* near Hainan Island (China) [21]. Also, the yellow oils citromycetin and 2,3-dihydrocitromycetin have been isolated from a marine derived *Penicillium bilaii* [22]. The examples of other molecules that can also color fungal structures or be excreted as secondary metabolites include tetrahydroauroglaucin (yellow) and isodihydroauroglaucin (orange) which have been extracted from *Eurotium* sp., an isolate from leaves of the mangrove plant *Porteresia coarctata* [23]. The yellow compound flavoglaucin and the mycotoxin citrinin have been shown to be produced by the marine-derived fungus *Microsporum* sp. in Korea [24]. A fungus of the genus *Periconia* isolated from hypersaline environment (solar saline in Puerto Rico) subjected to high solar radiations has been shown to produce a still unidentified, and unusual blue pigment [25].

To date, most of the studies on the marine fungi have highlighted that these fungal genera and species are facultative and not obligate microorganisms. With regards to the industrial production of dyes, this may be considered as an advantage because strictly marine fungal species (able to grow only in the marine environment) are often difficult to culture at a large scale [19••]. The feature to culture at a large scale is highly required for the industrial production of biochemicals including the pigments. Ubiquitous strains including the members of the genera *Aspergillus* and *Penicillium* are frequently encountered in marine habitats and usually produce enough biomass for chemical studies or industrial exploitations. These two genera have been intensively investigated for decades in the quest for interesting secondary metabolites both from the terrestrial and the marine origin. Among these secondary metabolites, some are considered as new, although in many cases, they are biogenetically, closely related to natural products described previously from their terrestrial counterparts. For example, the so called novel yellow 2,3-dihydrocitromycetin from the marine-derived isolate of *Penicillium bilaii*, collected from the Australian Huon estuary (Port Huon, Tasmania) has also been identified from a soil isolate of *Penicillium striatisporum* [22]. Up-to-now a few unique colored compounds have not yet been found of their counterparts produced from the terrestrial isolates. The examples are the brown bisdihydroanthracenone derivative, eurorubrin or the new orange anthraquinone glycoside [3-O-(α-D-ribofuranosyl)-questin] from *Eurotium rubrum*, isolated from the inner tissue of the stem of the mangrove plant *H. tiliaceus* around Hainan Island (China). Seemingly, no terrestrial counterpart has been discovered yet for the new yellow compound dimethoxy-1-methyl-2-(3-oxobutyl) anthrakunthone produced by the mangrove's endophytic *Fusarium* sp. ZZF60 isolated from the South China Sea [26], or for the red alterporriols: K, L, and M from the endophytic *Alternaria* sp. found in the fruit of the mangrove's shrub *Aegiceras corniculatum* (Zhanjiang Guangdong, South China Sea) [20]. *Penicillium commune* G2M isolated from the mangrove plant *H. tiliaceus* (Hainan Island, China) has also been reported to synthesize a pale yellow oil charaterized as 1-O-(2,4-dihydroxy-6 methylbenzoyl)-glycerol [27], and *Penicillium* sp. JP-1 from the inner bark of an *Aegiceras corniculatum* tree collected in Fujian (China) has been claimed to produce a red pigment named penicillenone [28]. Finally, the yellow anthracene-glycoside asperflavin-ribofuranoside from the marine-derived fungus *Microsporum* sp. (Korea) [24] appears only to be produced by marine fungi.

Unique features of pigment producing marine fungi and the exploitation of their metabolic potential

Many marine fungi have been reported to be endophytes and to make associations with higher life forms (plants, algae, corals). Examples have shown that under these conditions the fungi may proceed to biochemically mimic the host organism [29]. This is not surprising since the fungi have to deal with the marine environment and the biological context of the host. Algae can then be considered as valuable sources for the isolation of pigment producing marine fungi to the

extent that many algae are pigmented. Therefore, the algicolous fungi may produce unusual and novel dyeing molecules. In addition, co-cultivation of marine fungi with other microorganisms from the same ecosystem has been proved to be successful in activating silent gene clusters to produce bioactive secondary metabolites [30••]. Even if the microorganism can be easily, genetically manipulated and simply scaled-up for metabolite production, the modification of cultivation parameters such as media composition can also possibly induce and regulate secondary metabolite biosynthesis [31]. Inactivation or enhancement of selected steps of a biosynthetic pathway by a chemical approach can then be an alternative tool to metabolic engineering, using mutations or genetic transformation techniques. One of the main advantages of using inhibitor and precursor feeding is that the genetic and epigenetic background of the cell remains unchanged. However, the overexpression of a transcriptional factor controlling a metabolic pathway can affect the expression of certain genes or modify some cellular processes. The use of such techniques requires a thorough knowledge of the biosynthetic pathways and the enzymes involved.

Conclusions

The current use and the potential of using filamentous fungi as pigment and natural colorant sources for food applications are promising considering the ever rising demand by the consumers to replace their synthetic counterparts. Filamentous fungi are readily available raw materials that can be tailored to make microbial cell factories for the production of food grade pigments because of their chemical and color versatility in their pigment profile, easier large scale controlled cultivation, and a long-term history of well known production strains for the production of a variety of other biochemicals including colorants. Emphasis has been put on the screening for specific pigments, such as natural blue, or red colorant for cochineal extract/carminic acid/carmine partial replacement by the food colorant industries. In this regard the HAQN pigments and/or novel chemical classes from marine pigment producing fungi could be an interesting avenue to be explored further. As in the case of other food additives and/or ingredients, all sources of natural pigments and colorants (plants, minerals, insects, microalgae, microorganisms) will coexist in the market, with market shares depending on consumer's expectations, industrial prices and availability.

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CHAPITRE 2

Production des pigments parmi le genre *Arthrobacter*

Les pigments jouent un rôle important dans les processus physiologiques et moléculaires des micro-organismes, par exemple le rôle protecteur vis-à-vis du rayonnement solaire, l'adaptation à un environnement extrême et la photosynthèse. Depuis plusieurs décennies, les pigments ont été utilisés comme un outil taxonomique pour l'identification et la classification des bactéries. Actuellement, en plus du rôle dans l'étude taxonomique, des micro-organismes produisant des pigments ont été largement étudiés par la fermentation et le génie biologique en raison de leur potentiel biotechnologique.

Avec l'intérêt croissant pour les pigments microbiens grâce à des facteurs tels que la production indépendante de la saison, de nouveaux micro-organismes à partir desquels les pigments peuvent être extraits facilement sont en cours d'évaluation. Aujourd'hui, les pigments microbiens de qualité alimentaire sont disponibles dans le commerce.

Dans la nature, un grand nombre de micro-organismes produisent des pigments par exemple des levures, des champignons, des algues et des bactéries. Le genre Arthrobacter est l'un parmi les multiples micro-organismes qui ont été décrits comme producteurs de pigments. En outre, les bactéries de ce genre produisent une grande variété de teintes et de structures de pigments, ce qui est particulièrement rare.

Article 3

☒ Bacteria belonging to the extremely versatile genus Arthrobacter as novel sources of natural pigments with extended hue range. Sutthiwong N., Fouillaud M., Valla A., Caro Y., Dufossé L.. Food Research International, 2014, 65, 156-162.

Le genre *Arthrobacter* constitue un groupe prédominant de micro-organismes de divers environnement comme la terre, l'air, les aliments (par exemple les produits laitiers), l'eau, l'huile, la saumure, les feuilles de tabac, la peau humaine, etc. Le genre *Arthrobacter* a été créé par Conn et Dimmick. Il appartient à la classe *Actinobacteria*, la sous-classe *Actinobacteridae*, l'ordre *Actinomycestales*, le sous-ordre *Micrococcineae*, et l'ordre *Micrococcaceae*.

La plupart des bactéries du genre *Arthrobacter* sont aptes à produire une gamme de pigments, bien que quelques espèces soient non pigmenté comme par exemple *Arthrobacter scleromae* sp.nov., and *Arthrobacter albus* sp. nov.. Plusieurs études expérimentales précédentes ont montré que les pigments produits par les bactéries de ce genre ont différentes teintes, par exemple jaune (*Arthrobacter flavus* sp. nov. *Arthrobacter aurescens*), vert (*Arthrobacter crytallopoietes* sp. nov.), bleu (*Arthrobacter oxydans*, *Arthrobacter atrocyaneus*) et rouge (*Arthrobacter roseus* sp. nov., *Arthrobacter agilis*).

Depuis longtemps, le genre *Arthrobacter* retrouvé dans diverses sources a été étudié, et de nombreuses souches rapportées ont des colonies colorées. La purification et la caractérisation de leurs pigments n'ont pas été systématiquement effectuées pour acquérir des connaissances sur les structures chimiques et les rôles des pigments dans ces souches. Cependant, suffisamment de données sont disponibles et sont présentés ci-après pour les molécules jaunes, bleues, vertes et rouges, afin de révéler le potentiel de ces bactéries. Cette revue vise à mettre en lumière le potentiel des pigments issus d'espèces d'*Arthrobacte*r et d'autres études devront être menées pour mettre en œuvre les méthodes de production et d'extraction.

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Bacteria belonging to the extremely versatile genus *Arthrobacter* as novel source of natural pigments with extended hue range

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ABSTRACT

The genus *Arthrobacter* is a predominant group of bacteria isolated from various sources such as soil, air, foods (e.g. dairy products), water and plants. Species of this genus have been found in extreme environments e.g. oil, brine, mural paintings, clinical specimen, sewage and activated sludge. Most bacteria in the genus *Arthrobacter* produce pigments in a broad range of hues e.g. orange and yellow (riboflavin, carotenoids), blue and green (indigoidine, indochrome and derived salts), and red (porphyrins, carotenoids), pigments which are listed and reviewed in the present work. However, the purification and characterization of these pigments have not been extensively and fully investigated. Nowadays, pigment producing microorganisms have been increasingly of interest in many scientific disciplines and applications have broadened in the industry because of their biotechnological advantages. Consequently, more complete studies on pigments production by the genus *Arthrobacter* may be worthwhile to conduct as there is a high possibility to discover novel sources of food colorants.

1. Introduction

Pigments play an important role in physiology and molecular processes of microorganisms e.g. solar radiation protectant, adaptation to extreme environment and photosynthesis. Since several decades, pigments have been used as a taxonomic tool for the identification and classification of bacteria (Kuhn & Starr, 1960; Schippers-Lammertse, Muijsers, & Klatser-Oedekerk, 1963). Diversity of pigments depends on the differences in their chemical structures and the presence of specific chromophores (Hui & Hurlbert, 1979). Currently, besides the role in taxonomic study, pigment-producing microorganisms have been widely investigated through fermentation and bioprocess engineering due to their biotechnological potential (Dufossé, 2006; Dufossé, Fouillaud, Caro, Mapari, & Sutthiwong, 2014; Dufossé et al., 2005; Venil, Zakaria, & Ahmad, 2013).

With the growing interest in microbial pigments because of factors such as production regardless of season and geographical conditions, novel microorganisms from which pigments can be easily extracted are being assessed. Nowadays, microbial food-grade pigments are commercially available (Dharmaraj, Ashokkumar, & Dhevendaran, 2009; Dufossé, 2006; Marova, Haronikova, Petrik, Dvorakova, & Breierova, 2012). Furthermore, in the industry, there are possibilities to produce some microbial pigments for applications, for example, in animal feeds, cosmetics, pharmaceutical and textile (Dufossé et al., 2005; Higuera-Ciapara, Felix-Valenzuela, & Goycoolea, 2006; Venil & Lakshmanaperumalsamy, 2009). In nature, a great number of microorganisms e.g. yeast, fungi, algae and bacteria produce pigments. Nevertheless, appropriate species should satisfy some criteria: i) ability to use a variety of C and N sources, ii) tolerance to growth conditions (e.g. pH, temperature, nutrient concentration), iii) providing expected color at reasonable yield, iv) non-toxic and non-pathogenic product, and v) simple extraction of pigment from cell biomass if not excreted (Brunke et al., 2010; Hailei, Ping, Yufeng, Zhifang, & Gang, 2012; Zhou et al., 2009).

Over other pigment producing microorganisms, bacteria offers certain distinctive advantages, owing to their short life cycle, non-sensibility to season and climate, potentiality to produce pigments of different colors and shades, easier scale-up production of pigments, etc. Some examples of the pigment producing bacterial species include *Flavobacterium* sp. which produces the yellow pigment zeaxanthin, *Agrobacterium aurantiacum* (pink-red pigment, astaxanthin), *Micrococcus* sp. (various colored pigments, carotenoids), *Pseudomonas aeruginosa* (blue-green pigment), *Serratia marcescens* (red pigment), *Chromobacterium* sp. (violet pigment) and *Rheinheimera* sp. (blue pigment) (Venil et al., 2013).

The genus *Arthrobacter* is one among diverse microorganisms that have been found to produce pigments. In addition, bacteria of this genus produce a great variety of pigment hues and structures, which is quite uncommon (*Streptomyces* species have the same 'chemical plasticity'). The genus *Arthrobacter* constitutes a predominant group of

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microorganisms from various environments such as soil, air, foods (e.g. dairy products), water, oil, brine, tobacco leaves, human skin, mural paintings, clinical specimen, sewage and activated sludge (Chang et al., 2007; Chen et al., 2009; Ding, Hirose, & Yokota, 2009; Funke et al., 1996; Gupta, Reddy, Delille, & Shivaji, 2004; Irlinger, Bimet, Delettre, Lefèvre, & Grimont, 2005; Kallimanis et al., 2009; Kim et al., 2008; Koch, Schumann, & Stackebrandt, 1995; Margesin, Schumann, Spröer, & Gounot, 2004; Osorio, Barja, Hutson, & Collins, 1999; Reddy, Aggarwal, Matsumoto, & Shivaji, 2000; Sguros, 1955; Stackebrandt, Koch, Gvozdiak, & Schumann, 1995; Wauters, Charlier, Janssens, & Delmee, 2000). At the present time, 80 species of this genus have been described in the taxonomy (Euzéby, 2012).

The genus *Arthrobacter* was created by Conn and Dimmick (1947). It belongs to the *Actinobacteria* class, the *Actinobacteridae* subclass, the *Actinomycetales* order, the *Micrococcineae* suborder, and the *Micrococcaceae* family. The members of this genus are Gram-positive, aerobic, catalase-positive, and produce little or no acid from glucose, and also have a rod–coccus morphological cycle which depends on a growth period (rod shape in young cultures and coccus shape in older cultures). The genus *Arthrobacter* has been mainly divided into two groups on the basis of their peptidoglycan structure and menaquinone composition (Chang et al., 2007; Reddy et al., 2000; Schleifer & Kandler, 1972). In group I, the species of *Arthrobacter* contain the A3α peptidoglycan variant, in which murein is cross-linked by interpeptide bridges involving monocarboxylic L-amino acids, or glycine, or both, as observed in most species of *Arthrobacter* including the type strain of the genus, *Arthrobacter globiformis* (Reddy et al., 2000; Stackebrandt, Fowler, Fiedler, & Seiler, 1983). Within group II, species possess the A4α peptidoglycan variant, in which the peptidoglycan type is L-Lys-Ala-Glu or L-Lys-L-Glu such as those present in *Arthrobacter nicotianae*, *Arthrobacter uratoxydans*, *Arthrobacter protophormiae*, *Arthrobacter sulfureus* (Funke et al., 1996; Schleifer & Kandler, 1972; Stackebrandt et al., 1995). *Arthrobacter rhombi*, a new species isolated from Greenland halibut with A4α peptidoglycan, was also included in *Arthrobacter* group II (Osorio et al., 1999). Phylogenetic studies indicated that some *Micrococcus* species such as *Micrococcus luteus*, *Micrococcus agilis* and *Micrococcus lylae* were intermixed with species from the genus *Arthrobacter* (Stackebrandt et al., 1995) and detailed phylogenetic analysis along with phenotypic similarities have resulted in the reclassification of *M. agilis* as *Arthrobacter agilis* (Koch et al., 1995). This genus also comprised a number of other species for which the chemotaxonomic data are lacking, e.g. *Arthrobacter picolinophilus*, *Arthrobacter siderocapsulatus*, *Arthrobacter mysorens* and *Arthrobacter radiotolerans* (Keddie, Collins, & Jones, 1986). Nevertheless, two of these four species have been reclassified later: *Arthrobacter picolinophilus* as *Rhodococcus erythropolis* and *A. radiotolerans* as *Rubrobacter radiotolerans* (Koch et al., 1995; Suzuki, Collins, Iijima, & Komagata, 1989). In addition, numerous other species have been now excluded from the genus *Arthrobacter* because of meso-diamino pimelic acid (DAP) or LL-DAP content in the peptidoglycan (Reddy et al., 2000; Suzuki et al., 1989).

Most of the bacteria in the genus *Arthrobacter* produce a range of pigments, although a few species are un-pigmented e.g. *Arthrobacter scleromae* sp. nov., *Arthrobacter histidinolovorans* and *Arthrobacter albus* sp. nov. (Ding et al., 2009; Huang et al., 2005; Kim et al., 2008; Wauters et al., 2000). Several previous experimental studies showed that pigments produced by bacteria from this genus have various hues, e.g. yellow (*Arthrobacter flavus* sp. nov., *Arthrobacter aurescens*, *Arthrobacter ilicis*, *Arthrobacter citreus*, *A. nicotianae*, *Arthrobacter arilaitensis*, *Arthrobacter protophormiae*, *A. uratoxydans*, *Arthrobacter sulfureus*, *A. luteolus* and *A. mysorens*) (Chang et al., 2007; Irlinger et al., 2005; Kallimanis et al., 2009; Reddy et al., 2000), green (*Arthrobacter crystallopoietes* sp. nov., *A. pyridinolis*, *A. viridescens*) (Ensign & Rittenberg, 1963; Knackmuss & Beckmann, 1973; Kolenbrander & Weinberger, 1977; Schippers-Lammertse et al., 1963), blue (*Arthrobacter oxydans*, *A. atrocyaneus*, *Arthrobacter polychromogenes*) (Knackmuss, Cosens, & Starr, 1969; Kuhn & Starr, 1960) and red (*Arthrobacter roseus* sp. nov., *A. agilis*, *A. hyalinus*) (Fong, Burgess, & Barrow, 2001; Kojima et al., 1993; Reddy et al., 2000). The nature of these compounds is still partly unknown, although some literature reported that yellow pigments belonging to the carotenoid family play an important role in physiological and molecular processes of these microorganisms such as: photosynthesis, survival to oxidative damage and resistance to UV-radiations (Dieser, Greenwood, & Foreman, 2010; Fong et al., 2001; Liu, Gai, Tao, Tang, & Xu, 2012).

Since a long time the genus *Arthrobacter* found in various sources has been studied, and numerous reported strains have colored colonies. The purification and characterization of their pigments were not systematically conducted to gain knowledge about the chemical structures and the roles of the pigments in these strains. However enough data are available and are presented hereafter for yellow, blue, green and red molecules, in order to reveal the great potential of these bacteria. This review is only suggesting the potential of *Arthrobacter* pigments and other studies must be conducted to implement the production and extractions methods.

2. Yellow pigments

2.1. Riboflavin

Riboflavin, also known as vitamin B2 (Fig. 1), is yellow or orange-yellow in color. It plays a role in the biosynthesis of flavocoenzymes as it is the direct precursor of the 2 flavocoenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These are essential cofactors for a multitude of mainstream metabolic enzymes that mediate hydride, oxygen, and electron transfer reactions. Only plants, fungi, and microorganisms can synthesize riboflavin, whereas higher animals, including human, must obtain it through diet.

Some previous studies declared that *Arthrobacter* sp. isolated from soil e.g. *Arthrobacter globiformis* excreted a yellow pigment during exponential growth which was identified as riboflavin, $C_{17}H_{20}N_4O_6$, 7,8-dimethyl-10-(1′-D-ribityl) isoalloxazine (Hamm & Decker, 1980; Veldkamp, Venema, Harder, & Konings, 1966; Yamane, Nakamra, Okamoto, Oshima, & Kato, 1995).

Yamane et al. (1995) investigated the riboflavin production by an *Arthrobacter* sp. mutant resistant to 5-fluorouracil by using a jar fermentor and the optimal conditions in pH and dissolved oxygen concentration (DO) for the production were identified. The maximum production of riboflavin depended on both (i) pH of a culture medium which contained L-glutamic acid, glucose, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and yeast extract and (ii) DO level during the logarithmic growth phase. Maximum production of riboflavin was 160 mg/L under controlled

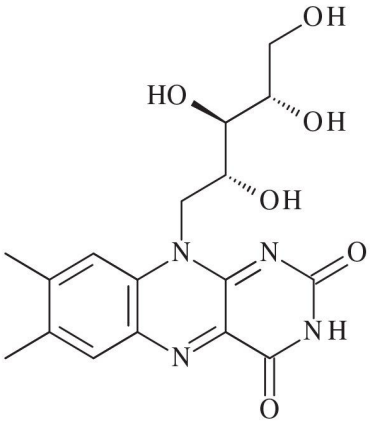

Fig. 1. Chemical structure of riboflavin produced by *Arthrobacter globiformis*.

conditions (600 rpm agitation, aeration rate of 1.0 L/min, and pH 7.0 at 30 °C).

The role of riboflavin in cells of *Arthrobacter* sp. has not been clearly revealed. Only few studies showed that it may play a role as a factor conditioning the growth and the development in natural ecosystems. According to Donderski (1991) and Donderski and Nowacka (1992), the heterotrophic bacteria isolated from the littoral zone of mesotrophic lake produce more riboflavin in the autumn than in the spring.

2.2. Carotenoids

In 1975, one of the yellow-colored psychrophilic bacterium *Arthrobacter glacialis* isolated from morainic mud was investigated (Arpin, Faisson, Norgard, Borch, & Liaaen-Jensen, 1975; Arpin, Liaaen-Jensen, & Trouilloud, 1972). Column chromatography technique was applied to purify crude extracts, followed by thin layer chromatography on silica gel. Electronic and mass spectroscopy, including ^{1}H NMR technique, were also used. These techniques revealed two yellow C_{50} –carotenoids with molecular formula $C_{50}H_{72}O_2$: the bicyclic decaprenoxanthin (Fig. 2 (compound 1)) and the monocyclic *A.g* 470 (Fig. 2 (compound 2)).

Arthrobacter flavus sp. nov., a psychrophilic bacterium isolated from a cyanobacterial mat sample from a pond in Antarctica, was taxonomically characterized and its pigments were analyzed (Reddy et al., 2000). The results showed that the yellow pigment was insoluble in water but soluble in methanol and gave visible absorption spectrum with absorption maxima at 410, 440 and 470 nm. The absorption spectra demonstrated carotenoid characteristics of this pigment but the authors did not identify the compound.

More recently, Galaup et al. (2007) reported that *Arthrobacter arilaitensis*, one of the major bacterial species found at the surface of smear-ripened cheeses, produces a yellow pigment which was tentatively identified as a carotenoid mixture. Furthermore, the carotenoids excreted by this strain may belong to the C_{50}-subfamily according to the study of Monnet et al. (2010) about the carotenogenesis gene cluster.

Although the role of carotenoids produced by the genus *Arthrobacter* has not been distinctly revealed, one function of carotenoids in microorganisms is to serve as membrane integrated antioxidants, protecting cells from oxidative stress (Krinsky, 1989; Liu et al., 2012).

3. Blue pigments

3.1. Indigoidine

Two blue-colored pigment producing bacteria from the genus *Arthrobacter*, *A. atrocyaneus* sp. nov. and *A. polychromogenes*, were isolated and identified (Kuhn & Starr, 1960; Kuhn, Starr, Kuhn, Bauer, & Knackmuss, 1965; Schippers-Lammertse et al., 1963). Since blue pigments, specifically indigoidine, are not frequently produced by bacteria, the pigments of these two strains were likewise investigated for their biology and chemistry.

Indigoidine is a brilliant blue pigment produced by *A. atrocyaneus* and *A. polychromogenes*, which is insoluble in water. The color of indigoidine produced by these two strains is violet-blue when it is finely powdered, while its color shows a brilliant reflection like copper when it is pressed on a porous plate (Kuhn et al., 1965).

The chemical structures of indigoidine and derivatives described by Kuhn *et al.* (Kuhn et al., 1965) are shown in Fig. 3. Indigoidine (compound 1a), $C_{10}H_8N_4O_4$, is 5,5′-diamino-4,4′-dihydroxy-3-3′-diazadiphenoquinone-(2,2′). By heating with 6 N HCl, indigoidine yields a hydrolysis product (compound 2a), $C_{10}H_6N_2O_6$, which has been identified as 4,5,4′,5′-tetrahydroxy-3-3′ -diazadiphenoquinone-(2,2′). This hydrolysis product formed intermediately a monopotassium salt, which appears green (Fig. 3, compound 2 with $R_2 = K^+$).

Derivatives of indigoidine were identified as diacetylindigoidine (compound 1b), $C_{14}H_{12}N_4O_6$, 4,5,4′,5′-tetrahydroxy-3-3′ -diazadiphenoquinone-(2,2′); the diacetyl derivative of 2a (compound 2b), $C_{14}H_{10}N_2O_8$, 4,4′-dihydroxy-5,5′-diacetoxy-3,3′-diazadiphenoquinone-(2,2′); the hexaacetoxy derivative of compound 2a (compound 3), $C_{22}H_{20}N_2O_{12}$, 2,5,6,2′,5′,6′-hexaacetoxy-3,3′-bipyridyl; and the dimethoxy derivative of compound 2a (compound 2c), $C_{12}H_{10}N_2O_6$, 4,4′-dihydroxy-5,5′-dimethoxy-3,3′-diazadiphenoquinone-(2,2′).

Another strain in the genus *Arthrobacter* which produces the blue pigment related to indigoidine is *A. crystallopoietes*; although colonies of *A. crystallopoietes* are brilliant green in color (Ashworth, 1974; Ensign & Rittenberg, 1963; Knackmuss & Beckmann, 1973; Kolenbrander & Weinberger, 1977).

The pigment of *A. crystallopoietes* is a monopotassium salt of 4,5,4′,5′-tetrahydroxy-3,3′-diazadiphenoquinone-(2,2′), $C_{10}H_5N_2O_6K$. This pigment is similarly a monopotassium salt of the hydrolysis product of indigoidine produced by *A. atrocyaneus* and *A. polychromogenes*. It is sparingly soluble in cold water and more soluble in hot water, with a blue color which fades to pale yellow after a few minutes. With acetic anhydride, the potassium salt reacts to a golden yellow diacetyl derivative which is identical to diacetyl derivative of hydrolyzed indigoidine (Fig. 3 (compound 2b)).

Beside of *A. atrocyaneus*, *A. polychromogenus* and *A. crystallopoietes*, the strain *Arthrobacter oxydans* has been reported to produce a blue pigment which also relates to indigoidine (Knackmuss & Beckmann, 1973).

During degradation of nicotine, *Arthrobacter oxydans* excretes a blue pigment which is derived from pyridine-2-6-diol and pyridine-2,3,6-triol (Knackmuss, 1973). This pigment was separated into two major components by using cellulose thin layer chromatography. Their chromophores are anions of the diazadiphenoquinone indigoidines, nicotine

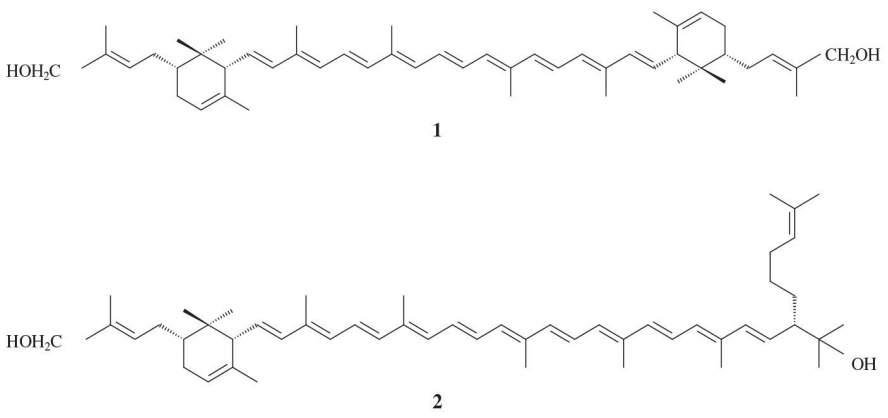

Fig. 2. Chemical structures of carotenoids produced by *Arthrobacter glacialis*. 1: decaprenoxanthin; 2: monocyclic *A.g.* 470.

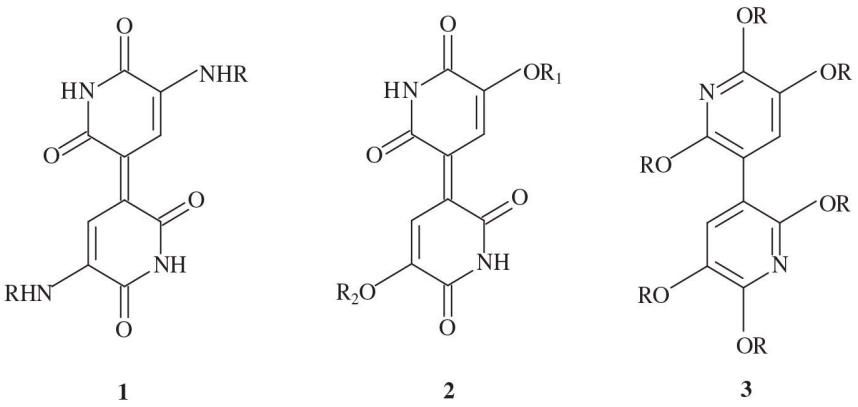

Fig. 3. Chemical structures of indigoidine and its derivatives produced by *Arthrobacter* species (e.g. *Arthrobacter atrocyaneus*, *Arthrobacter polychromogenes*). 1. a: R = H; 1. b: R = CH_3CO. 2. a: R1 = R2 = H; 2. b: R1 = R2 = CH_3CO; 2. c: R1 = R2 = CH_3; 2. d: R1 = H; R2 = OH. 3. R = CH_3CO [monopotassium salt of 2a is a green compound].

blue I (Fig. 3 (compound 2a)) and nicotine blue II (Fig. 3 (compound 2d)). The formation of nicotine blue I was observed during the stationary growth phase of *A. oxydans*, while nicotine blue II was already exhibited during the exponential growth phase in culture broth. The crystalline pigment consisted in a large amount (approximately 80%) of nicotine blue I. It was identified as a potassium salt by flame photometry. The acid form of nicotine I was identified with authentic 4,5,4′,5′-tetrahydroxy-3,3′-diazadiphenoquinone-(2,2′) by UV, nuclear magnetic resonance spectra and by the formation of derivatives [i.e. 4,4′-dihydroxy-5,5′- diacetoxy-3,3′ -diazadiphenoquinone-(2,2′) and 2,5,6.2′,5′,6′-hexaacetoxy-bipyridyl-(3,3′)]. A minor component, nicotine blue II ($C_{10}H_6N_2O_5$), approximately 20%, was found in the crystalline pigment obtained from solid medium. This pigment showed increased water solubility and mainly diffused into agar. In contrast to nicotine blue I, it was exceedingly labile at pH ≤ 5 and exhibited increased stability at higher pH values.

The specific function of the blue pigment indigoidine produced by *Arthrobacter* species is still unknown; although, there is a recent study on the role of this pigment in plant-pathogenic enterobacterium *Erwinia chrysanthemi* according to Reverchon, Rouanet, Expert, and Nasser (2002). The authors reported that the indigoidine production of this bacterium provided an increased resistance to oxidative stress, signifying that indigoidine may protect the bacteria against the reactive oxygen species generated during the plant defense response.

3.2. Indochrome

Apart from indigoidine, another chromophore of the water-soluble pigment produced by *A. atrocyaneus* and *A. polychromogenes* was identified as indochrome (Knackmuss, 1973; Knackmuss et al., 1969). This pigment was released into the liquid culture only by indigoidine-producing bacteria. It has been initially described by Knackmuss et al. (1969) who analyzed its chemical structure.

The four indochromes (indochrome A, indochrome BI, indochrome BII, and indochrome BIII) with chemical formula $C_{20}H_{23}N_3O_{12}$ have been reported as pigments excreted from *A. polychromogenes*. The analysis of their electron spectra in methanol demonstrated that indochrome A spectra corresponded to the spectrum of the model compound, 3-methyl-5-benzeneazo-2,6-dihydroxy-piridine, while indochrome BI and BII have been identified to be 3-α-D-ribopyranosyl-5-benzeneazo-2,6-dihydroxy-pyridine. The chemical structures of indochrome A and indochrome BII are shown in Fig. 4, compound 1 and compound 4, respectively. The pigment components of indochrome BI consist mainly in compound 2, and contain small quantities of compound 3 (Knackmuss, 1973).

4. Red pigments

4.1. Porphyrins

Porphyrins, a group of organic compounds, are highly colored cyclic tetrapyrrolic pigments, formed by the linkage of four pyrrole rings through methene (– HC–) bridges. The basic structure of a tetrapyrrole

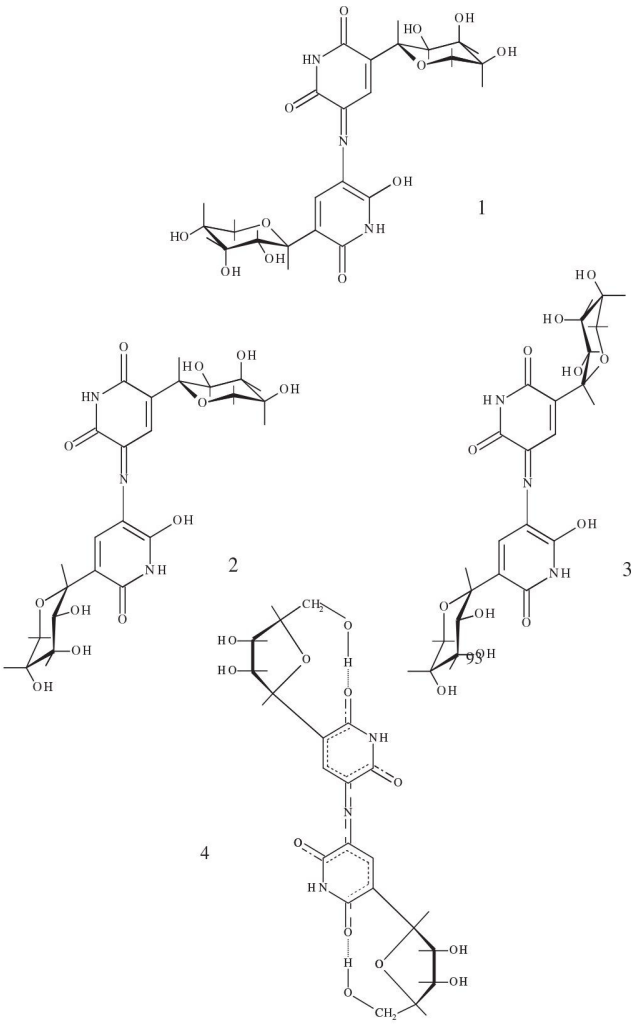

Fig. 4. Chemical structures of indochrome produced by *Arthrobacter polychromogenes*.

is four pyrrole rings surrounding a central metal atom and found in many organisms.

Porphyrin formation by the genus *Arthrobacter* e.g. *A. hyalinus*, *A. globiformis*, *A. aurescens*, *A. ramosus*, *A. cremeus*, *A. resinosus*, *A. isopropanolophila*, and *A. flavidus*, has been revealed (Kajiwara, Tokiwa, Takatori, & Kojima, 1995; Kojima, Fujiwara, & Maruhashi, 1982; Kojima et al., 1993; Kortstee, 1970).

According to Kortstee (1970), following absorption spectra measurement and chromatographic behavior of the red extracellular pigment in cultures of *Arthrobacter globiformis*, coproporphyrin III has been identified as the type of porphyrin produced by this bacterium. Lately, two new members from the genus *Arthrobacter*, *A. photogonimos* and *A. aurescens*, have been found to produce coproporphyrin (Scharf, Mamet, Zimmels, Kimchie, & Schoenfeld, 1994; Yang & Hoober, 1995). Following the isolation and the characterization of *A. aurescens*, the strain *A. aurescens* RS-2, was found to secrete a large amount of coproporphyrin III in response to aluminum, if present in cultures. Scharf et al. (1994) continued to examine the effects of this metal on the growth and the pigment formation of the strain. Aluminum delayed the growth of *A. aurescens*, while it enhanced the pigment formation in which coproporphyrin III was shown to be the main porphyrin produced by aluminum-exposed *A. aurescens* RS-2. The chemical structure of the red pigment coproporphyrin III, $C_{36}H_{38}N_4O_8$, produced by these *Arthrobacter* species is shown in Fig. 5.

In addition, another form of porphyrin has been characterized from pigments excreted by *Arthobacter hyalinus*, a bacterium isolated from soil, and cultured in a medium containing isopropanol as carbon source (Kajiwara et al., 1995; Kojima et al., 1993). This pigment has been identified as uroporphyrin III, $C_{40}H_{38}N_4O_{16}$ (Fig. 6).

In bacteria, porphyrins are essential parts of energy conserving electron transport chains and cofactors of various enzymes. Changes in environmental conditions usually induce an adaptation of the bacterial energy metabolism and often correspond with significant changes in cellular porphyrin levels (Schobert & Jahn, 2002)

4.2. Carotenoids

Recently, red-carotenoids accumulation in *Arthrobacter agilis*, a psychrotrophic bacterium isolated from Antarctic sea ice has been investigated (Fong et al., 2001). The results show that lowering the temperature used for cultivation resulted in a companion increase in carotenoid production. Maximum biomass accumulation occurred at 5–30 °C

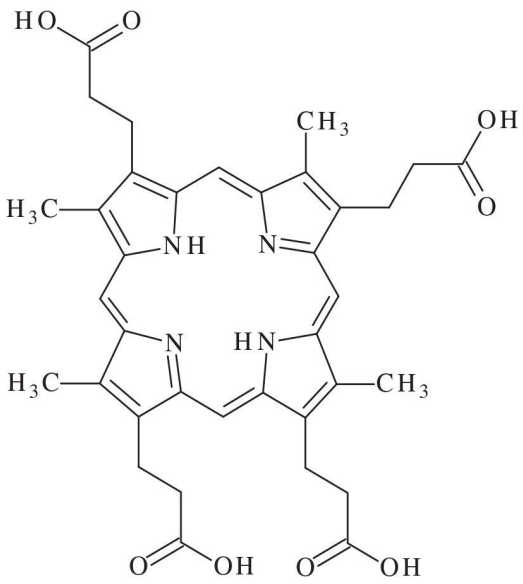

Fig. 5. Chemical structure of coproporphyrin III produced by *Arthrobacter aurescens*.

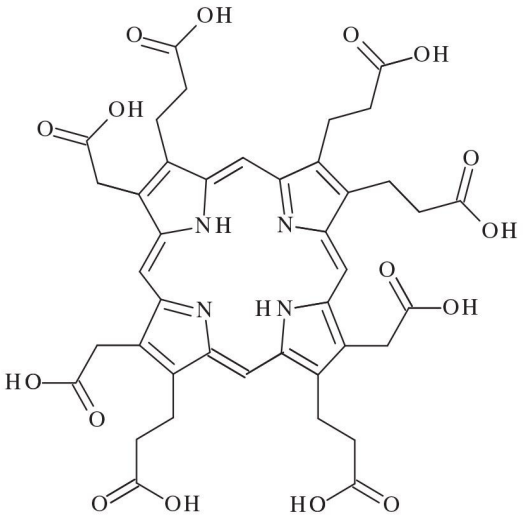

Fig. 6. Chemical structure of uroporphyrin III produced by *Arthrobacter hyalinus*.

with a tenfold reduction at 40 °C. The red carotenoids produced by the strain *A. agilis* MB813 have been identified as a series of geometrical isomers of the C_{50} carotenoid bacterioruberin (Fig. 7 (compound 1)), mono-, di-, and tetra-glycosylated derivatives of bacteriorubin, and a C_{50} hydrocarbon, tetra-anhydrobacterioruberin (Fig. 7 (compound 2)). These components were analyzed using a combination of UV–vis spectral data, HPLC retention times, molecular masses, and by a comparison to authentic bacterioruberin standard.

A. roseus sp. nov., a bacterium isolated from a cyanobacterial mat sample from a pond located in Antarctica, has been reported as a red carotenoid producer (Reddy, Prakash, Matsumoto, Stackebrandt, & Shivaji, 2003). The UV–Vis absorption spectra of chloroform/methanol extracts from this strain showed four absorption maxima, at 437, 467, 494 and 524 nm. Although the chemical structures of these red pigments were not fully investigated, their absorption spectra demonstrated characteristics of some carotenoid pigments.

According to Fong et al. (2001), changes in carotenoids production in response to growth temperature and salt concentration, provide insight in the adaptation of psychrotrophic and psychrophilic bacteria to the cold environment. Pigments may be associated with stabilization of cell membrane at low temperature. Shivaji and Ray (1995) have suggested that the survival of some microorganisms at low temperature may be enhanced by the ability of carotenoids to rigidify membranes. In addition, experimental results by Dieser et al. (2010) indicated that red-carotenoids increase the resistance of heterotrophic bacteria *Arthrobacter agilis* MB8-13 to environmental stress as being cryo- and solar radiation protectants.

5. Conclusion

Pigments are commonly used in numerous products, for example, food, coloring paint, animal feed, plastic, fabric, cosmetics, and medicine. Accordingly, synthetic and natural pigments have been immensely used in various industries. Pigments are responsible for the wide spectrum of colors in various objects. They are often associated with quality, attractive appearance, and sensory properties. In recent years, the utilization of natural pigments in manufacturing sectors e.g. animal feed, food, dye, textile, cosmetic and pharmaceutical has been increasing due to the demands by the consumers to replace synthetic dyes. Among the available sources, microorganisms seem to be a reasonable choice for colorant production due to biotechnological advantages e.g. (i) production regardless of seasons and geographical conditions, (ii) controllable and predictable yield.

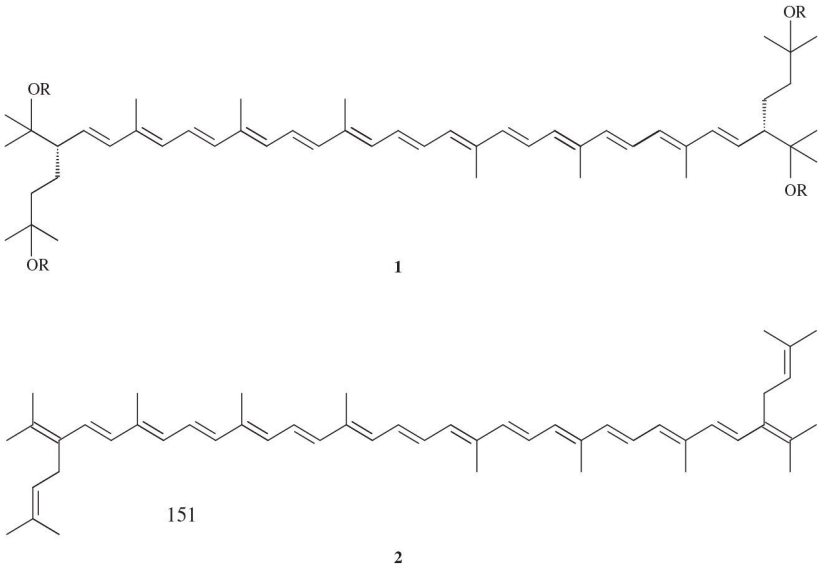

Fig. 7. Chemical structures of carotenoids produced by *Arthrobacter agilis*. 1. Bacterioruberin: R = H; a series of glycosylated derivatives: R = H, hexose or dihexose. 2. tetra-anhydrobacterioruberin.

The genus *Arthrobacter* is one among diverse microorganisms which has been found to produce great variety of pigment hues e.g. yellow, red, green and blue. These bacteria are commonly found in various sources such as soil, water, air and foods; as well as, in extreme environments such as sewage, radioactive waste tank and distilled water. By the advantages of this genus mentioned above, great industrial potential of pigment producing *Arthrobacter* strains should be included into the world's search for alternative microorganisms as novel sources of natural colorants. This potential is reinforced by the fact that many *Arthrobacter* species are inventoried in the lists of microbial species with technological beneficial role in fermented food products.

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CHAPITRE 3

Arthrobacter dans l'industrie laitière

Le lait est un aliment très nutritif car il contient naturellement toute une gamme de nutriments essentiels à la croissance, y compris les lipides, les protéines, les glucides, les vitamines, les minéraux et les acides aminés essentiels. En raison de la croissance démographique, l'augmentation des revenus, l'urbanisation et les changements dans les habitudes des consommation, la demande mondiale pour le lait et les produits laitiers ne cesse de croître. Grâce à des procédés de fabrication, des matières premières et des ingrédients, de nombreux produits dérivés du lait peuvent être créés afin de satisfaire cette exigence.

En plus de l'excellente source pour l'alimentation humaine, le lait a été décrit comme un environnement idéal pour la survie et la croissance d'une grande variété de micro-organismes. Des changements d'activités biochimiques dans les communautés microbiennes au cours de l'utilisation des nutriments conduisent aux différentes caractéristiques du lait et des divers produits laitiers. De nos jours, les micro-organismes sont largement utilisés dans l'industrie alimentaire pour la production de divers types de régimes alimentaires en raison de leurs propriétés souhaitables et variables.

Arthrobacter est un genre de bactéries largement répandu dans la nature, principalement dans le sol. A l'heure actuelle, environ 80 espèces de ce genre ont été décrits dans la taxonomie. Certaines de ces espèces d'Arthrobacter sont rencontrées dans le lait ou l'environnement des ateliers et sont souhaitables lors de la transformation de produits laitiers.

Article 4

☒ Commercial applications of versatile bacteria belonging to the genus A*rthrobacter* in milk and dairy products. Sutthiwong N., Dufossé L.. (Soumission prévue à une revue internationale).

Arthrobacter est un genre de bactéries largement répandu dans la nature, principalement dans le sol. Comme pour plusieurs bactéries du sol, *Arthrobacter* sp. est métaboliquement polyvalent, produit des enzymes différentes permettant sa croissance dans une grande variété de substrats; par conséquent, plusieurs espèces d'*Arthrobacter* peuvent connaître des applications dans de nombreuses industries.

En ce qui concerne l'impact positif des micro-organismes sur l'industrie laitière, cet article vise à mettre l'accent sur les bactéries polyvalentes appartenant au genre *Arthrobacter* et leurs applications dans le lait et les produits laitiers commerciaux (fromages par exemple).

En particulier dans l'industrie laitière, *Arthrobacter* sp. est souvent associé d'une manière compatible avec la production de fromage, car il est considéré comme l'un des micro-organismes principaux responsables des propriétés organoleptiques des fromages, c'est à dire la couleur et de la saveur. En outre, la capacité du genre *Arthrobacter* pour produire des enzymes actives à froid est importante pour la fabrication de produits laitiers dans des conditions modérées afin d'éviter les changements dans les propriétés organoleptiques et la perte de valeur nutritionnelle à température ambiante.

Les données rapportées dans la présente étude montrent distinctement les aspects bénéfiques de certains *Arthrobacter* sp. ainsi que leurs significations dans l'industrie laitière afin de motiver de nouvelles recherches sur le genre *Arthrobacter* en raison de la demande mondiale croissante pour le lait et les produits laitiers.

Commercial applications of versatile bacteria belonging to the genus *Arthrobacter* in milk and dairy products

Short title:

Applications of *Arthrobacter* bacteria in the dairy industry

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Abstract

Arthrobacter is a genus of bacteria widely distributed in nature, primarily in soil. Resembling many soil bacteria, *Arthrobacter* sp. is metabolically versatile, leading to produce different enzymes providing its growth in a wide variety of substrates. Accordingly, several *Arthrobacte*r species have likely been taken this advantage for applying in many industries, particularly in the dairy industry where *Arthrobacter* sp. consistently associates with the production of cheeses since it is considered as a major microorganism responsible for the organoleptic properties of cheeses i.e. color and flavor. Besides, the ability of *Arthrobacter* sp. to produce cold-active enzymes important for the dairy product manufacturing under mild conditions in order to avoid the changes in organoleptic properties and the loss of nutritional value at ambient temperature. Data reported in the present review distinctly shows the beneficial aspects of *Arthrobacter* sp. and their significances in the dairy industry in order to motivate new investigations of the genus *Arthrobacter* owing to the growing demand for milk and dairy products.

Keywords: *Arthrobacter* sp.; versatile bacteria; dairy products

1. Introduction

Milk is a highly nutritious food since it naturally contains a whole range of nutrients essential for growth including fats, proteins, carbohydrates, vitamins, minerals and essential amino acids. All of milk obtained from a variety of animal sources has a nearly neutral pH and a high water activity (Wijesinha-Bettoni, Burlingame, 2013). By reason of population growth, rising incomes, urbanization and changes in consumption habit, global demand for milk and dairy products is continuing to grow. Via manufacturing processes, commodity and ingredients products obtained from milk can be created in order to fulfill this requirement.

Besides the excellent source for human nutrition, milk has been referred as a great environment for the survival and growth of a large variety of microorganisms. Some of these nutrients are able to use directly by all microorganisms, while others are provided following the types of microorganisms presented such as lactose, which is not utilized by many bacteria, and large molecules e.g. proteins and lipids, which must be broken down by enzymes for allowing sustained microbial growth (Ledenbach, Marshall, 2010). Changes of biochemical activities in microbial communities during the nutrient utilization lead to the different characteristics of milk depending on the period of these activities; therefore, microbes advantageously associate with milk and dairy product technology. With accumulating knowledge about the functionality of microorganisms, it became less complicated to apply them for biotechnological processes. Nowadays, microorganisms are widely used in the food industry to produce various types of diets due to their desirable properties. In the dairy industry, the utilization of microorganisms has been increasingly interested since it is an important part for quality controlling as well as for quality developing.

Arthrobacter is a genus of bacteria widely distributed in nature, generally in soil. Bacteria of the genus *Arthrobacter* are Gram-positive, catalase-positive, obligated aerobic and asporogenous bacteria displayed a coryneform morphology, which belong to the family *Micrococcaceae* of *Actinomycetales* (Keddie, Collins, Jones, 2006). At the present time, approximately 80 species of members in this genus have been described in the taxonomy (Euzéby, 2012). These *Arthrobacter* species enclose a variety of desirable members of technological relevance to dairy products such as *Arthrobacter arilaitensis* and *Arthrobacter bergeri*, which response for color development in smear-ripened cheeses (Irlinger, Bimet, Delettre, Lefèvre, Grimont, 2005).

Regarding the positive impact of microorganisms on the dairy industry, this review aims to focus on the association of versatile bacteria belonging to the genus *Arthrobacter* with their significances in the applications to commercial milk and dairy products.

2. Occurrence of *Arthrobacter* sp. in raw milk

The origin of microorganism occurrence in milk and dairy products varies in the type of products and the mode of production and processing. After synthesizing within the mammary gland of healthy animals, milk is virtually sterile. Besides this stage, microbial contamination in raw milk may generally occur from three principal sources i.e. interior of the udder, exterior of the udder, and from the equipment used for milk handling and storage (Monsallier, Verdier-Metz, Agabriel, Martin, Montel, 2012; Quigley et al., 2013; Touch, Deeth, 2009). The presence of microorganisms in dairy products, beyond the contamination of raw milk which is used as a raw material, can derive from the processing and post-processing lines (Dogan, Boor, 2003; Lücking, Stoeckel, Atamer, Hinrichs, Schulz, 2013; Samelis, Kakouri, Rogga, Savvaidis, Kontominas, 2003). Members of the genus *Arthrobacter* are commonly present in raw milk as a primary microflora, and are believed to enter from the milking equipment as well as the teat surface (Champagne, Laing, Roy, Mafu, 1994; Verdier-Metz, Michel, Delbès, Montel, 2009). Outdoor environments such as air in the stable and milking parlor and hay were also identified to be the sources of *Arthrobacter* sp. (Vacheyrou, Normand, Guyot, Cassagne, 2011)

Despite microbial composition of milk have been investigated by clarifying several groups of microorganisms, there are few studies identifying the presence of *Arthrobacter* sp. in raw milk; furthermore, detail of species diversity of these bacteria has been much less studied. In most cases, due to the diversity of the microbial flora of raw milks contributes to the differences in organoleptic characteristics such as flavor and color among raw milk cheeses and pasteurized milk cheeses, *Arthrobacter* sp. was identified as one of microorganisms appeared in raw cow milk (Vacheyrou et al., 2011). Using culture-independent method, 2 species namely *Arthrobacter arilaitensis* and *Arthrobacter psychrolactophilus* were identified (Quigley et al., 2013). To our knowledge, considering the occurrence level of *Arthrobacter* sp. in milk (goat) taken from the same herd at different periods over an entire lactation year, only Callon et al. (2007) detailed that less than 1% of these bacteria loaded among 41 different species.

3. Significance of *Arthrobacter* sp. for the dairy industry

Although *Arthrobacter* sp. are generally defined as soil microorganisms, new species have been also isolated from various sources, for example, sea water, glacier ice, wastewater sediment and animal (Chen et al., 2009; Ding, Hirose, Yokota, 2009; Gupta, Reddy, Delille, Shivaji, 2004; Osorio, Barja, Hutson, Collins, 1999). Due to several species of *Arthrobacter* sp. are psychrophilic, the extreme resistance to dryness and starvation elucidate the predominance of these bacteria. Furthermore, they also exhibit the nutritional versatility to degrade different compounds from both nature and synthetic (Keddie

et al., 2006). As the result of their metabolic diversity, *Arthrobacter* sp. has been taken advantages of this ability to use in various branches of industry and biotechnology, especially in the dairy industry (Fig. 1).

3.1 Association of Arthrobacter sp. with cheese production

Cheeses can be considered as a complex microbial ecosystem, consisted of various species of microorganisms including yeasts and bacteria originating from milk, starter cultures and cheese-making environments. In consequence of the microbial interactions among these microorganisms during cheese production processes, organoleptic and textural properties of cheese will be developed (Brennan et al., 2002; Irlinger, Mounier, 2009). These biochemical changes are noticeable in smear-ripened cheeses due to the diversity and abundance of microorganisms on its surface. Surface ripening commences with the growth of yeasts, which metabolize lactic acid and produce growth factor useful to bacteria. The utilization of lactic acid by yeasts progressively leads to the deacidification of the cheese surface. When pH increases to near a neutral value, less-tolerant acid bacteria begin to grow and eventually cover the entire cheese surface. Besides yeasts, this microbial mat is mainly composed of *Brevibacterium linens* and other coryneform species, including *Microbacterium* sp., *Corynebacterium* sp. and *Arthrobacter* sp. (Bockelmann, Hoppe-Seyler, Krusch, Hoffmann, Heller, 1997; Irlinger et al., 2005).

The presence of *Artrhobacter* strains in surface-ripened cheeses, as well as in mold surface-ripened cheeses, has recently been reported; for example, *Arthrobacter nicotianae*, *Arthrobacter arilaitensis* and *Arthrobacter bergerei* (Irlinger et al., 2005; Valdès-Stauber, Scherer, Seiler, 1997). From several varieties of Austrian cheeses, numerous isolates were preliminarily identified as *Arthobacter globiformis* whereas most *Arthrobacter* strains could not be identified to any known species (Eliskases-Lechner, Ginzinger, 1995). Since the colonies of *Arthobacter* sp. isolated from cheeses commonly display yellow colors and these bacteria have been found at different stages of ripening, it is then assumed that *Arthrobacter* sp. is one of the major microorganisms contributing to the typical texture, flavor and color properties of the final product. (Feurer, Vallaeys, Corrieu, Irlinger, 2004; Galaup et al., 2007; Larpin-Laborde et al., 2011).

3.1.1 Implication of Arthrobacter sp. on cheese color

Similarly a large number of other products, the appearance of cheeses is a key element of a consumer's purchasing decision due to it appears as a sign of quality including an attractive aspect. In this respect, the color is a trace associated with many qualities of cheeses such as flavor, naturalness, or

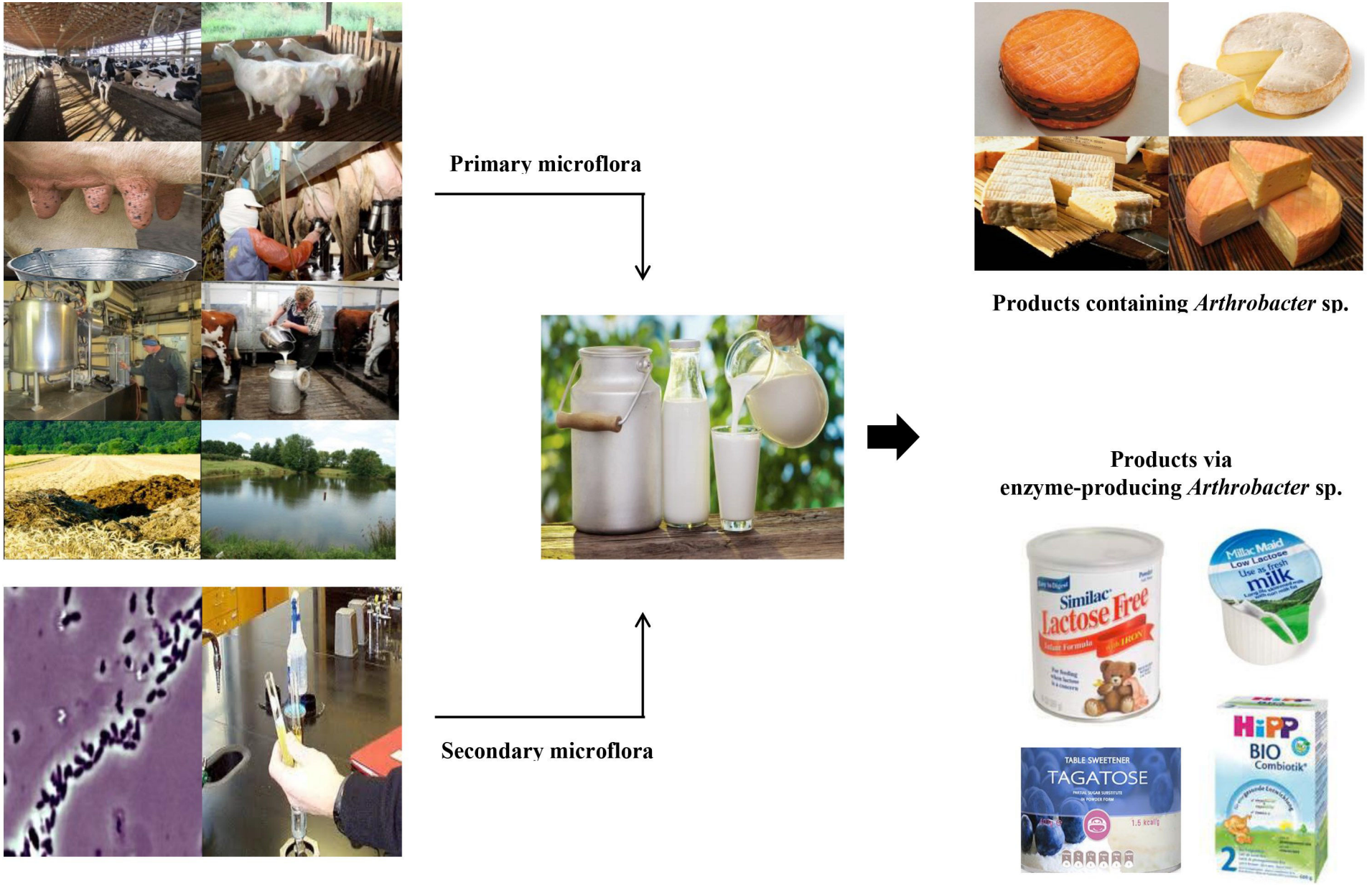

Fig. 1 The potential sources of the occurrence of *Arthrobacter* sp. in milk and significant benefits of *Arthrobacteria* sp. in the dairy industry.

maturity (Dufossé, Galaup, Carlet, Flamin, Valla, 2005). This is markedly occurs in red-smear ripened cheeses, which are characterized by an orange to orange-reddish color of microbial mat on the cheese rinds. The color is due essentially to carotenoids, in combination with other pigments, produced by the cheese microflora during ripening (Guyomarc'h, Binet, Dufossé, 2000; Leclercq-Perlat, Oumer, Bergere, Spinnler, Corrieu, 2000; Mounier et al., 2008). Besides *Brevibacterium linens*, which in the past was considered to be a sole microorganism responsible for the color development, the other main types discovered to be present in the surface flora of cheeses are cream-colored and yellow-pigmented coryneform (Brennan et al., 2002; Eliskases-Lechner et al., 1995; Fontana, Cappa, Rebecchi, Cocconcelli, 2010; Mounier et al., 2005; Rea et al., 2007). According to Bockelmann (2002), the color development of typical light-brown cheese was attributed to the interactions between yellow-pigmented *Arthrobacter* sp. and other microorganisms while the orange pigments *B. linens* were found lesser important, confirming the results of Eliskases-Lechner and Ginzinger (1995). other microorganisms while the orange pigments *B. linens* were found lesser important, confirming the results of Eliskases-Lechner and Ginzinger (1995).

Although the occurrence *Arthrobacter* sp. on the surface ripening of smear cheeses was often stated, only a few studies have been taxonomically identified these isolates. Several *Arthrobacter* strains were previously found on the surfaces of different cheese varieties i.e. *Arthrobacter aurescens*, *Arthrobacter citreus*, *Arthrobacter globifomis*, *Arthrobacter protophormiae*, *Arthrobacter uratoxydans* and *Arthrobacter variabilis* (Eliskases-Lechner et al., 1995). More recently, some strains among *Arthrobacter* species namely *Arthrobacter rhombi* and *Arthrobacter sulfureus* were isolated from Limburger cheeses, smear-ripened semi-soft cheeses widely produced in Belgium, Germany and Netherlands (Bockelmann, Willems, Neve, Heller, 2005). *Arthrobacter nicotianae* is also shown to be predominant bacteria found from different cheeses such as Tilsit, Chaumes, Limburger and Romadour cheeses (Bockelmann, 2002). Using both cultivation and rDNA-based methods, strains of *Arthrobacter arilaitensis* have been reported to be one of the major bacterial species found at the surface of soft red-smear cheeses e.g. French Livarot, Munster, and Reblochon (Feurer, Irlinger, Spinnler, Glaser, Vallaeys, 2004; Irlinger et al., 2005). Besides *A. arilaitensis*, the presence of *A. bergerei* also exhibited in these French cheeses (Irlinger et al., 2005).

Despite available information regards to the diversity of bacteria belonging to the *Arthrobacte*r sp. isolated from a variety of smear cheeses, very little is known about the coloration of cheese by these bacteria. Consequently, for a purpose of providing information to cheese-manufacturers when applying *Arthrobacter* sp. as a part of ripening flora, different aspects related to the cheese coloration should be investigated e.g. the variation of pigment production among strains and factors affecting the pigmentation including physical and chemical factors, as well as the microbial interactions between

Arthrobacter sp. and other microorganisms found on cheeses. Furthermore, the ability of *Arthrobacter* sp. to produce pigments is probably resulting in the application of these bacteria as sources for the production of food colorants.

3.1.2 Implication of Arthrobacter sp. on cheese flavor

The biochemical changes among diverse complex microbial communities, which occur especially during ripening, involve the conversion of milk fat, carbohydrate and protein to a very wide range of flavor compounds by three principal metabolic pathways i.e. lipolysis, glycolysis and proteolysis. These pathways require several different enzymes obtaining from milk endogenous enzymes, clotting enzymes, manufacturing and ripening microbial enzymes (Deetae, Bonnarme, Spinnler, Helinck, 2007). The degradation of amino acid by microbial enzymes, which are mainly deaminase, transaminase, decarboxylase, lyase and dehydratase, yields aldehydes, amines, acids, alcohols and sulphur compounds while the breakdown of fatty acid will deliver methyl ketones, secondary alcohols and esters (Ardo, 2006; Marilley, Casey, 2004). Depending on a variety of microflora presented during the ripening process, the substrates will be metabolized by a number of pathways to numerous compounds contributing to cheese flavor or off-flavors (McSweeney, Sousa, 2000).

Among the microorganisms found from the surface of cheeses, very few have been observed the ability of bacteria to produce flavor compounds, especially of which by bacteria belonging to the genus *Arthrobacter*. Since *Arthrobacter* sp. have been recently revealed as one of the major microorganisms related to the properties of cheeses, to our knowledge, only two species of the genus *Arthrobacter* were investigated their association with the flavor formation in cheeses. *A. nicotianae*, a typical microflora of German Tilsit cheese, were found to be the most significant for the development of a typical odor, in the combination with *B. linens* (Bockelmann, 2002) Without other microorganism, a more or less typical odor was also developed in liquid model system with only these two species (Bockelmann, Hoppe-Seyler, 2001). Several strains of *A. arilaitensis* isolated from French smear-ripened cheeses were used to investigate their ability to produce volatile aroma compounds. Two strains, namely *A. arilaitensis* Mu107 and *A. arilaitensis* Po102, have been shown to be capable of producing various volatile compounds e.g. aldehydes, esters, ketones, alcohols and sulphur compounds (Deetae et al., 2007).

Up to now a number of *Arthrobacter* sp. isolated from smear cheeses have been discovered. Not only the yellow-pigmented *Arthrobacter* sp, a large proportion of grey-white or cream-colored *Arthrobacter* isolates were also found on the surface of cheeses (Bockelmann, 2002; El-Erain, 1969). These pale-colored isolates are assumed to not responsible for the cheese coloration but for the development of flavor; in addition, they probably play a major role in the cheese flavor formation.

Accordingly, to achieve a better knowledge associated with the cheese flavor, the investigation mentioned bacteria of the genus *Arthrobacter* may be required. The utilization in the cheese industry of bacteria belonging to this genus with specific flavor-forming abilities will be later considered as a promising tool to respond to the developing and controlling quality for organoleptic properties of cheese products.

3.2 Association of Arthrobacter sp. producing enzymes with milk and dairy products

In the dairy industry, enzymes are widely used for the production and the improvement of product quality, as well as for the innovation for new products. The industrial demand for new enzyme sources with different characteristics and low cost production increases a number of scientific researches related to the isolation and selection of new microorganisms. Among a diversity of microorganisms, psychrophilic bacteria are currently intensively interested due to their synthesis of cold-active enzymes. With a high possibility to apply at low temperature, especially in food industry such as in the processing of milk and dairy products, these enzymes are increasingly of commercial interest. Although several cold-active enzymes have been discovered, β-galactosidase, commonly known as lactase which hydrolyzes lactose to glucose and galactose, seems to be the significant food-industrial enzyme because of its ability to degrade lactose for several purposes (Nakagawa, Ikehata, Myoda, Miyaji, Tomizuka, 2007).

β-galactosidase can be used, by removal lactose from refrigerated milk, to produce lactose-free milk and other related products e.g. cheeses, yoghurt, butter and ice cream for people who are lactose intolerant (Bialkowska, Cieśliński, Nowakowska, Kur, Turkiewiez, 2009). Furthermore, different oligosaccharides derived from lactose, as well as lactulose and D-tagatose, can be synthesized using β-galactosidase as biocatalyst. All of them display functional properties as prebiotics and as non-caloric sweeteners (Delzenne, 2003; Tang et al., 2011; Wanarska, Kur, 2011).

The Antarctic *Arthrobacter* strain, namely *Arthrobacter* sp. 20B, was found to produce β-galactosidase, which was optimally active at pH 6.0 - 8.0. Unlike an ideal enzyme, which should work well at 4 - 8^{o}C for treating milk, the optimal active temperature of the enzyme produced by strain 20B was 25^{o}C (Bialkowska et al., 2009). Several strains of *Arthrobacter* sp. isolated from soil, including *Arthrobacter psychrolactophilus* and *Arthrobacter chlorophenolicus*, have been discovered to produce β -galactosidase (Nakagawa et al., 2007; Wanarska et al., 2011). As a result of this study, cold-active β-galactosidase produced by *A. psychrolactophilus* strain F2 is likely a suitable enzyme using under low temperature condition due to its activity was highest at 0^{o}C while its optimum temperature and pH were 10^{o}C and 8.0, respectively.

As the demand for milk and dairy products is growing due to the population growth, rising

incomes, urbanization and changes in consumption habit, the utilization of enzymes in this industry may be increasingly needed in order to produce different products and also to develop and/or control the product qualities. Although only few species of the genus *Arthrobacter* were currently reported to produce cold-active enzymes important for the dairy industry, *Arthrobacter* sp. possibly provides advantages to use as potential producers for these enzymes to fulfill the commercial requirements.

4. Conclusions

Milk typically contains a variety of bacteria, of which some are beneficial such as those preserving milk through products of fermentation e.g. cheeses and yoghurt. Members of the genus *Arthrobacter* are among the major groups of microorganisms used in the dairy industry. While approximately 80 species of *Arthrobacter* sp. isolated from various environments were taxonomically identified, only few species have been recognized to apply in the dairy industry. Almost of *Artrhobacter* sp. associated with the application for dairy products were isolated from cheeses, which are responsible for the coloration and flavor formation of cheeses, whereas a small number of *Arthrobacter* sp. were isolated from soil, which are employed as producers for β-galactosidase used to hydrolize lactose in milk leading to gain other related products. Regarding these beneficial aspects derived from *Arthrobacter* sp., including pigmentation, flavor formation and enzyme production, *Arthrobacter* sp. is a promising potential bacterial genus important for the dairy industry. However, the better understanding in its roles related to these aspects is still required in order to enlarge the available benefits, especially those for cheese production. Meanwhile, the isolation for obtaining new strains to use as pigment, flavor compound and enzyme producers will possibly lead to complete the industrial requirements, not only of the dairy industry but also of other industries.

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CHAPITRE 4

Production des pigments chez d'*Arthrobacter arilaitensis*

Les fromages à croûte lavée sont des produits laitiers importants économiquement, qui subissent une succession de divers micro-organismes au cours du processus de production, principalement dans la coagulation du lait et l'affinage. La surface des fromages à croûte lavée est un tapis microbien, qui présente généralement une couleur orange-rougeâtre, composée d'une grande diversité de bactéries et de levures. La couleur de la surface du fromage à croûte lavée est définie comme étant l'un des principaux attributs qui affecte une acceptation par les consommateurs de ces fromages en raison de la représentantion potentielle de nombreuses qualités, comme par exemple la maturité, le naturel, la saveur et la propreté. La coloration des fromages à croûte lavée semble être un phénomène complexe en raison des interactions fonctionnelles entre les micro-organismes sur la croûte de fromage.

Les pigments générés par les bactéries isolées de frottis à la surface du fromage ont été peu étudiés, exception faite pour celles de *Brevibacterium linens*. Pendant longtemps, cette bactérie a été considérée comme le micro-organisme majeur unique responsable pour le développement de la couleur à la surface des fromages en raison de sa capacité à produire des caroténoïdes oranges. Toutefois, au cours des dernières années, avec les méthodes plus précises pour l'identification taxonomique, d'autres micro-organismes ont été signalés comme aussi importants que *B. linens* pour la maturation.

Les bactéries *Arthrobacter* ont récemment été isolées sur des fromages à croûte lavée et ont été décrits comme certains des principaux micro-organismes impliqués dans la pigmentation jaune de ces fromages en raison de sa couleur globale caractéristique et sa présence dans les différentes étapes jusqu'à la fin de l'affinage du fromage. *Arthrobacter arilaitensis* est une parmi les bactéries *Arthrobacter* obtenues sur des fromages à croûte lavée, mais a été rapportée comme étant une espèce bactérienne dominante sur la surface du fromage, montrant la capacité de production de pigment jaune. Par conséquent, nous avons porté notre attention sur *Arthrobacter arilaitensis*, les bactéries caroténogènes trouvées dans des fromages à croûte lavée, des produits laitiers largement consommés en Europe.

Article 5

☒ Production of carotenoids by *Arthrobacter arilaitensis* strains isolated from smear-ripened cheeses. **Sutthiwong N.**, Dufossé L.. FEMS Microbiology Letters, 2014, doi: 10.1111/1574-6968.12603.

Les caroténoïdes constituent un groupe de pigments terpéniques jaune à rouge-orange synthétisés par une large variété de plantes et de micro-organismes. Ils gèrent des fonctions biologiques importantes comme la protection contre les dommages photo-oxydatifs ou le rôle de collecteurs de lumière ou encore en tant que stabilisants de membrane cellulaire. Parmi les sources naturelles, les micro-organismes semblent être une alternative commerciale importante dans la production de caroténoïdes en raison du potentiel des biotechnologies. Les caroténoïdes produits par des micro-organismes sont acceptés comme additifs alimentaires, y compris comme colorants, dans de nombreux pays. Une grande variété de pigments caroténoïdes naturels peut être prévue selon la recherche en cours sur de nombreux micro-organismes. Cependant, certaines bactéries ont reçu peu de considération même si elles sont souvent présentes dans les aliments consommés ordinairement, par exemple les bactéries utilisées pour le fromage.

Arthrobacter arilaitensis est l'un des principaux micro-organismes trouvés à la surface des fromages, remarquablement dans les fromages à croûte lavée, où il est supposé être responsable de la pigmentation jaune de la croûte de fromage. La caractérisation de la synthèse de pigments par *A. arilaitensis* fournirait des informations utiles pour expliquer le processus de coloration des fromages. Jusqu'à présent, la recherche scientifique sur la synthèse des caroténoïdes par *A. arilaitensis* en est encore à ses balbutiements, et un article analysant la production de caroténoïdes par cette espèce n'existe pas. Afin de fournir des informations décrivant la production de pigments caroténoïdes par *A. arilaitensis*, plusieurs aspects complémentaires ont été étudiés.

FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

Production of carotenoids by *Arthrobacter arilaitensis* strains isolated from smear-ripened cheeses

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Arthrobacter arilaitensis; carotenoids; smear-ripened cheese; smear microflora.

Abstract

Arthrobacter arilaitensis is one of the major microorganisms responsible for the coloration of cheese surface, particularly in smear-ripened cheeses. This study investigated the occurrence of pigment synthesis among *A. arilaitensis* strains in several aspects covering (1) UV-Vis absorption spectra and HPLC chromatograms of pigment extracts, (2) diversity of pigment production among strains, (3) influence of light on the production of pigment, and (4) kinetic of pigment synthesis. Based on absorption spectra and HPLC analysis, the 14 *A. arilaitensis* strains studied could be divided into two groups depending on their ability to produce carotenoids, carotenoid-producing, and nonpigmented strains. The methanolic extracts prepared from eight carotenoid-producing strains contained at least four carotenoids represented mainly as polar molecules. The diversity of pigment concentrations among these strains was low, with carotenoids ranging from 0.40 to 0.76 mg L^{-1} culture and specific productivities from 0.14 to 0.25 mg pigment per g dry biomass, under light condition. When cultivating these *A. arilaitensis* strains under darkness condition, carotenoid biosynthesis was lower within a 0.17–0.25 mg L^{-1} range. The pigment production time curve of a representative colored *A. arilaitensis* strain displayed a sigmoid shape which paralleled cell growth, probably indicating a growth-associated pigmentation.

Introduction

Carotenoids are a group of yellow to orange-red terpenoid pigments synthesized by a wide variety of plants, algae, and microorganisms. More than 750 different carotenoids have been currently characterized from natural sources (Netzer *et al.*, 2010). They handle significant biological functions as protectants against photo-oxidative damages or light harvesting molecules or as membrane stabilizers (Vershinin, 1999; Takano *et al.*, 2005).

The global market value of commercially used carotenoids in 2010 was estimated at nearly $1.2 billion, with a possibility to rise to $1.4 billion by 2018 (BCC Research, 2014). Facing the increasing demand of these molecules, new sources of carotenoids have widely been explored. Among the natural sources, microorganisms seem to be an important commercial alternative in the production of carotenoids due to biotechnological strengths, for example, production regardless of season and geographical conditions, and controllable and predictable yield. Carotenoids produced by microorganisms are accepted as food additives including colorants in many countries (Mortensen, 2006). However, some bacteria have been given little consideration even though they have been usually found in foods, that is, bacteria used for cheese smear ripening. Consequently, we drove our attention to *Arthrobacter arilaitensis*, the carotenogenic bacteria found in smear-ripened cheeses, dairy products widely consumed in Europe.

Arthrobacter arilaitensis is one of the major microorganisms found at the surface of cheeses, remarkably in smear-ripened cheeses, where it is assumed to be responsible for yellow pigmentation of the cheese's rind because of its characteristic overall color and its involvement at the different stages of cheese ripening (Feurer *et al.*, 2004; Galaup *et al.*, 2007; Larpin-Larborde *et al.*, 2011). According to Monnet *et al.* (2010), the yellow pigmentation

of *A. arilaitensis* is probably due to the C50 carotenoid, decaprenoxanthin (Fig. 1). The characterization of pigment synthesis by *A. arilaitensis* would provide information to explain the coloration process of cheeses. Besides its conventional use in cheese manufacturing, this molecule could afterward promote acceptability for ingredients extracted from bacteria which would lead to the enlargement of a variety of carotenoids available as food additives as well as for other purposes, for example, investigation of health benefits of C50 carotenoids (Heider *et al.*, 2014; Yatsunami *et al.*, 2014).

Until now, scientific research about carotenoids synthesis by *A. arilaitensis* is still in its infancy, and a report analyzing the production of carotenoids by this species is missing. To provide information describing the production of carotenoid pigments by *A. arilaitensis*, four complementary aspects were investigated including (1) UV-Vis absorption spectra and HPLC chromatograms of pigment extracts, (2) diversity of biomass and pigment productions among strains, (3) influence of light on the biosynthesis of pigment, and (4) kinetic of pigment synthesis.

Materials and methods

Bacterial strains and culture medium

Fourteen *Arthrobacter arilaitensis* strains isolated from smear-ripened cheeses were used in this study. The strains isolated for years from many cheeses were kindly provided by the Institut National de la Recherche Agronomique (INRA), France. Usually stored at −80 °C, they were maintained during this study on milk ingredient-based agar, stored at 4 °C and subcultured monthly.

Three different types of bacterial culture media were used to cultivate *A. arilaitensis* in this study including two types from commercial brands, that is, tryptic soy broth (TSB, BD) and brain heart infusion (BHI, BD) and, as the third medium, a milk ingredient-based medium prepared according to Guyomarc'h *et al.* (2000). The milk ingredient-based medium containing 5 g casamino acids (BD Difco), 1 g yeast extract (BD Bacto), 5 g NaCl, 20 g D-glucose (Fisher Scientific), and 1 g KH_2PO_4 (Fisher Scientific) per liter of deionized water was prepared. Before sterilizing at 121 °C, 15 min, the pH of the medium was adjusted to 7.0 ± 0.2.

Cultures of *A. arilaitensis* strains were grown in 250-mL Erlenmeyer flasks filled with 50 mL of medium. Flasks were inoculated with 1% 72-h-old preculture (v/v) and incubated at 25 °C with 150 r.p.m. agitation using INFORS HT incubator. To study the effect of light on biomass and pigment productions, two series of cultures were incubated either in daylight or in the darkness.

Biomass and pigment quantitation

Dry biomass determination

Biomass produced by *A. arilaitensis* has been presented in this study in a form of dry matter content quantified using oven dry method (AOAC, 1990).

Pigment extraction and quantitation

As well as the quantitation of dry biomass, *A. arilaitensis* cells were obtained from cultures after centrifugation. Cells were then extracted with 8 mL of 99.9% methanol (Carlo Erba) and blended to prevent clotting. Samples were then wrapped with aluminum foil to protect from direct light, and the extraction was performed under 100 r.p.m. agitation, until cells were bleached. Suspension was centrifuged (6000 ***g***, 15 min), and then supernatant separated from cell debris, and extraction repeated until supernatant and residual cell pellet were colorless. The absorbance at 440 nm was measured using a UV/Vis spectrophotometer (UV-1800, Shimadzu) after purifying the extract by further centrifugation at 10 000 ***g*** for 15 min.

The concentration of pigments (mg pigments L^{-1}) was finally estimated according to the Beer–Lambert law. As we were unable to determine ourselves the specific absorption coefficients of expected decaprenoxanthin in methanol, previously published data of 1940 L g^{-1} cm^{-1} were eventually applied (Weeks *et al.*, 1980).

HPLC analysis

Methanol extracts were evaporated to dryness under vacuum at 55 °C in a Büchi rotavapor. High-performance liquid chromatographic (HPLC) analysis was performed with a HPLC device by Dionex-Ultimate 3000 (Thermo Fisher Scientific, France). For separation, a LichroCART

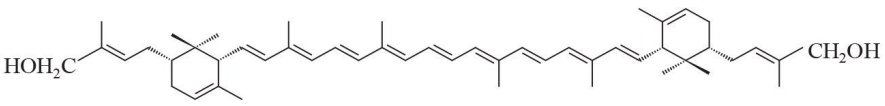

Fig. 1. Decaprenoxanthin ($C_{50}H_{72}O_2$), the C50 carotenoid probably produced by *Arthrobacter arilaitensis*.

RP-18 column (250 × 4 mm, 5 μm, Merck) was applied. Column temperature was set to 30 °C, and separation achieved using (A) methanol and (B) methyl *tert*-butyl ether as mobile phases. Elution from the column was isocratically carried out from 100% (A) for 25 min then a gradient was established over the next 45 min with (B) to a final ratio of 70 : 30 (A : B), followed by a further elution for 15 min with 70 : 30 (A : B). The column was then returned to the initial conditions and equilibrated over 20 min. A flow rate of 0.5 mL min^{-1} was employed. Monitoring, data recording, and processing were driven with the CHROMELEON 7 software (Dionex).

Growth and pigment production modeling

The sigmoidal function established by Gompertz (Zwietering *et al.*, 1990) was adjusted to experimental data obtained from the time course monitoring of dry biomass and carotenoid production in a culture of *A. arilaitensis* AA015. Adjustment was made using nonlinear regression method on SIGMAPLOT software (Systat Software, Inc.).

Statistical analysis

The data were analyzed using SIGMAPLOT software (Systat Software, Inc.). The *t*-test was performed for comparing mean values of individual variable of each strain between the two conditions at 95% significance level. When comparing data of each variable among 14 strains, one-way analysis of variance (one-way ANOVA) was applied. The difference of considered variable was estimated by Tukey's HSD test according to an α-risk of 5%.

Results

Absorption spectra and HPLC profiles of *A. arilaitensis* extracts

After cultivation for 7 days (i.e. period already known through preliminary experiments as long enough to obtain the plateau phase), all *A. arilaitensis* strains were collected and extracted, and then, pigment production was determined. Several extracts prepared from different strains of *A. arilaitensis* were intense yellow while some were less yellow or colorless. Cell extracts from shake flask cultures were first analyzed for pigment components by scanning the absorbance behavior from 800 to 200 nm. On wavelength scan data (data not shown), the extracts of eight strains, that is, *A. arilaitensis* AA012, AA015, AA020, AA021, AA028, AA030, AA035, and CNRZ923 exhibited a peak with absorbances ranging from 320 to 500 nm while the extracts of the other six strains (AA009, AA014, AA017, AA025, AA036, and WS2230) did not present peak throughout all wavelengths.

Following UV/Vis spectrophotometer scanning, HPLC was performed. Figure 2 details the chromatographic profiles obtained with methanolic extracts of *A. arilaitensis* AA015 and AA025 at 440 nm, which are representatives for strains presenting or not presenting a peak while scanning the absorbance, respectively. A typical chromatogram of strain AA025 extract did not display any peak, which corresponded to the results of spectrophotometric analysis, in contrast to the extract of strain AA015 which distinctly exhibited several peaks.

The chromatographic profile of strain AA015 methanolic extract was complex with at least five different components (Fig. 2a). These peaks could be categorized into two groups according to the elution time. Group I contained peak 1, eluting at roughly 19 min, followed 1.5 min later by a small but marked peak (2). Following group I, peak 3, peak 4, and peak 5 representing the group II were later eluted, between 26 and 32 min. Similar chromatograms were achieved from all other pigmented seven strains (AA012, AA020, AA021, AA028, AA030, AA035, and CNRZ923) cultivated for 7 days under the same conditions and culture medium. Spectra of all detectable compounds were closely related to the characteristic carotenoid pigments spectra (Table 1).

In another experiment, the pigments of strains AA015 and AA035 were analyzed after cultivation either in TSB or BHI liquid media. Although the peak area of extracts prepared from cultures grown in TSB and BHI was lower than the other ones grown in milk ingredient-based medium, all samples presented similar chromatograms (data not shown).

Diversity of pigment production among carotenoid-producing strains

Concerning the ability of carotenoid production, only eight strains of *A. arilaitensis* were estimated for dry matter and carotenoid production. Dry biomass obtained from these strains varied from 2.58 to 3.84 g L^{-1}, which led to yields < 19.2% conversion of glucose supplied, and pigment quantification of the extracts showed carotenoid content ranging from 0.40 to 0.76 mg L^{-1}. The specific productions calculated from their pigment and biomass quantities were between 0.14 and 0.25 mg pigments per g dry biomass.

To focus on the pigment production by each *A. arilaitensis* among eight carotenoid-producing strains, statistical analysis was further performed (Fig. 3). Although the results of statistical analysis indicated six groups with significant differences, the diversity of carotenoid production among the eight strains was low because there were several groups which consisted of many common strains.

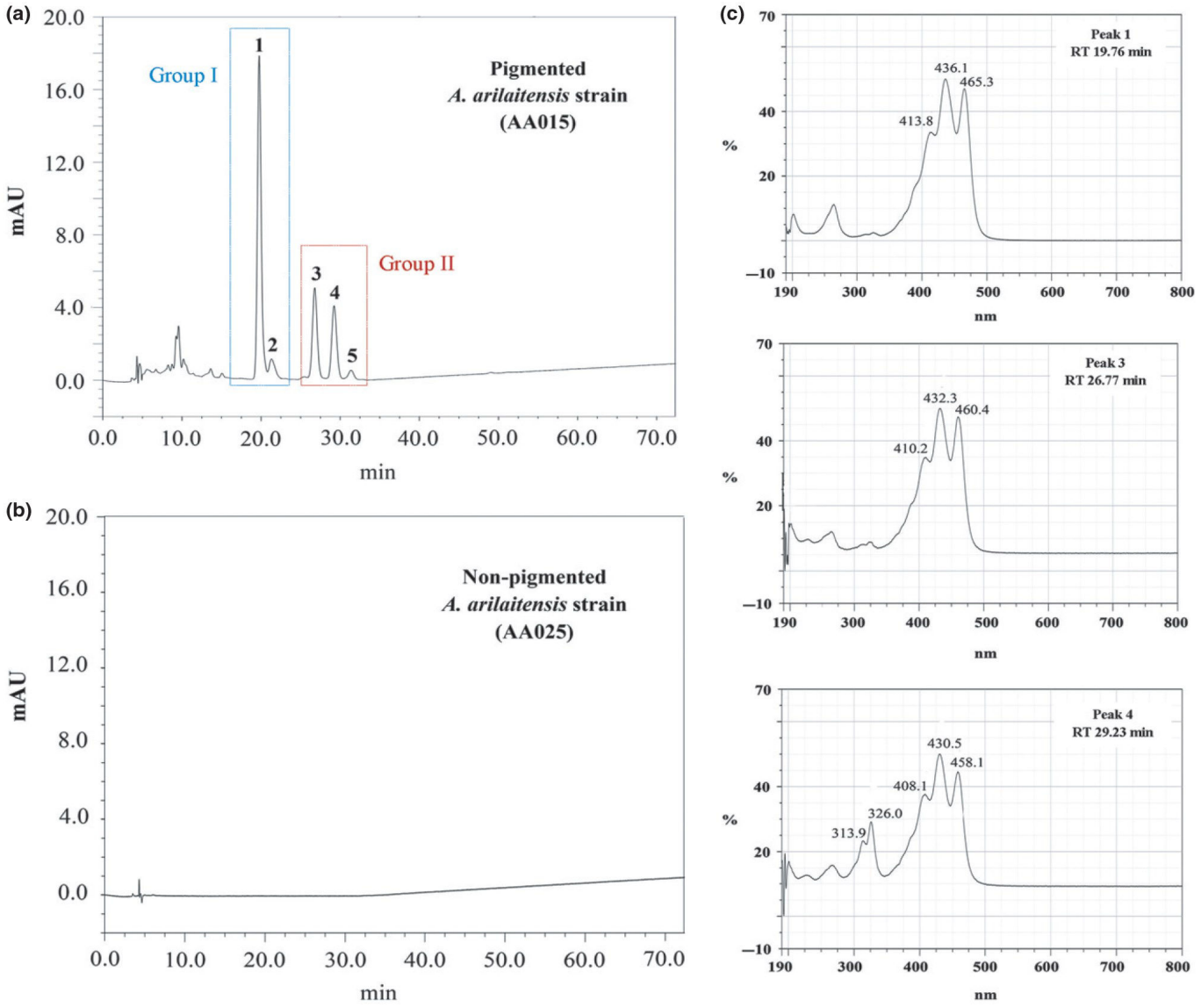

Fig. 2. HPLC chromatograms of extracts prepared from *Arthrobacter arilaitensis*. UV/Vis recorded at 440 nm. (a) strain AA015 methanolic extract; (b) strain AA025 methanolic extract; and (c) absorption spectra of principal peaks 1, 3, and 4.

Besides the milk ingredient-based medium, two other media, TSB and BHI, were also used to cultivate *A. arilaitensis* strains for comparing their pigment productions among these culture media. The results demonstrated that pigment production of cultures grown in milk ingredient-based medium, for seven among eight strains, was statistically significantly higher than these cultivated in TSB and BHI ($P < 0.05$). Except the strain AA012, the other strains were composed of AA015, AA020, AA021, AA028, AA030, AA035, and CNRZ923 showing a significant difference of pigment production between growing in milk ingredient-based medium and the two other media (data not shown).

Influence of light on biomass and pigment production of carotenoid-producing strains

After 15 days of simultaneous incubation in milk ingredient-based medium under either light or dark conditions (increased period of incubation due to unknown behavior of strains at dark), cultures of eight carotenoids-producing strains, AA012, AA015, AA020, AA021, AA028, AA030, AA035, and CNRZ923, were collected and their biomass and pigment productions further determined as previously described. When cultured in darkness, the dry biomass values ranged from 2.32 to 3.66 vs. 2.58 to 3.84 under light condition (data not shown); however, within

Table 1. Chromatographic and spectral properties of the major compounds detected in concentrated methanolic extracts prepared from *Arthrobacter arilaitensis* AA015

Strain	Group/peak no.	R_t (min)	λ_{max} (nm)	Data source
A. arilaitensis AA015	I*		413.8, 436.1, 465.3	This study
	1	19.76		
	II†		410.2, 432.3, 460.4	This study
	3	26.77		
	4	29.23	408.1, 430.5, 458.1	
Unknown yellow-pigmented bacterial strain isolated on smear-ripened cheeses		18.57	413.8, 437.9, 466.8	Galaup *et al.* (2005)
Unknown yellow-pigmented bacterial strain isolated on smear-ripened cheeses		20.9–21.1	412, 438, 466	Galaup *et al.* (2007)
		25.9	413, 438, 468	

*Peaks which were eluted between 19 and 21 min.
†Peaks which were eluted between 26 and 32 min.
Underlined values represent shoulders in the absorption spectra.

individual strains, there was not a statistically significant difference between the two conditions used for cultivation ($P < 0.05$) (Fig. 4a).

In contrast to the biomass production, carotenoids produced by these strains grown under light condition had higher pigment concentrations than the strains cultivated under dark condition. When cultivating in the light, carotenoid production varied as described previously between 0.40 and 0.76 mg L^{-1} with *A. arilaitensis* AA012 displaying the lowest carotenoids production, 0.40 mg L^{-1}, while *A. arilaitensis* AA028 presented the highest carotenoids content, 0.76 mg L^{-1}. The range of carotenoid production in the darkness was between 0.17 and 0.25 mg L^{-1}. After statistical analysis, carotenoids quantities produced by the same strains but grown in the different conditions were significantly different ($P < 0.05$) (Fig. 4b).

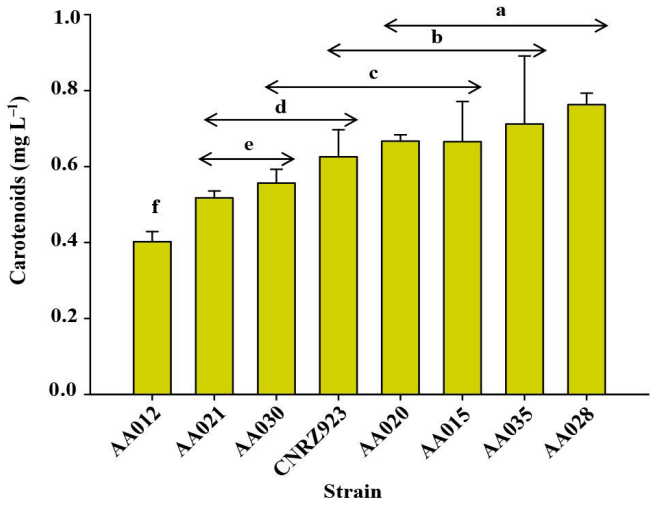

Fig. 3. Carotenoid production observed for eight strains of *Arthrobacter arilaitensis*. Each arrow represents the same letter above. Results with no letter in common were significantly different with a 95% confidence level. Standard deviation (SD) indicated in the columns was calculated from a set of data comprised of three independent experiments.

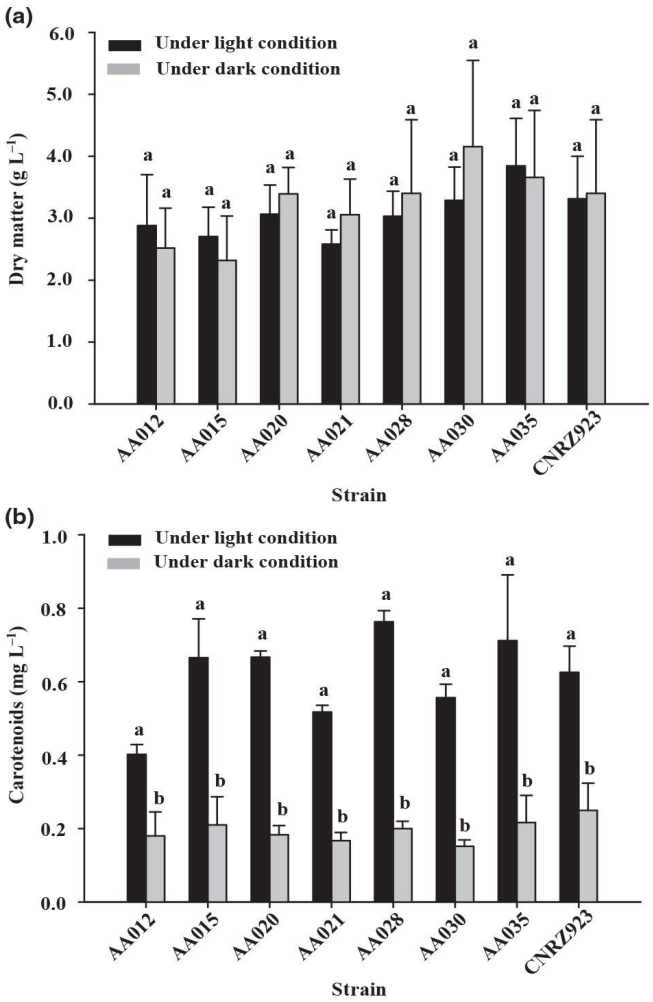

Fig. 4. Influence of light on biomass and carotenoids production by *Arthrobacter arilaitensis* strains; (a) biomass production and (b) pigment production. Results with no letter in common, between individual strains, were significantly different with a 95% confidence level.

The results led to conclude that light influenced the ability to produce pigments in all colored *A. arilaitensis* strains observed (AA012, AA015, AA020, AA021, AA028, AA030, AA035, and CNRZ923) but had no impact on their biomass production.

Kinetic of carotenoid production

Arthrobacter arilaitensis AA012 and AA015 were observed for biomass and pigment production in milk ingredient-based medium. After 7 days of cultivation, dry biomasses of the strains AA012 and AA015 were 1.62 and 1.47 g L^{-1}, respectively. Carotenoid production by *A. arilaitensis* AA012 was 0.29 mg L^{-1} while *A. arilaitensis* AA015 produced higher level of carotenoids than strain AA012, *c.* 1.5 folds, at 0.43 mg L^{-1}. There was a statistically significant difference of carotenoid concentration between these strains ($P < 0.05$), as a confirmation of the results from the diversity study previously monitored, even if in this second experiment, the overall biomass and carotenoid productions were lower for unknown reasons (hypotheses: (1) longer period of strain maintenance on agar plate before preparing the preculture, (2) sensitivity of *Arthrobacter* about the freshness of liquid medium for the cultivation that is number of days between sterilization and inoculation).

Figure 5 presenting *A. arilaitensis* AA015 as a representative colored strain cultivated in milk ingredient-based medium showed a 14-h lag phase of its biomass production and the exponential phase was during 14–48 h. After this, the biomass production was at a stationary phase until the end of observed time. For the pigment production, the data plots displayed a similar shape as the biomass production. Therefore, carotenoid synthesis seems closely related to growth. For a better description of this relationship between both responses, biomass and pigment production, the Gompertz model was applied. The model fitted the data with correlation coefficients of 98% and 97%, respectively, although the Gompertz model did not seem to be the best for curves with such a long transition phase. Model curves for dried matter and pigment production were then derived, leading to model dried biomass and pigment productivities (Fig. 5b). Pigment production was maximum at roughly 13 h after the growth reached the apex and stopped as growth ended. A typical production of primary metabolites could be used to explain the carotenoid production in *A. arilaitensis* AA015.

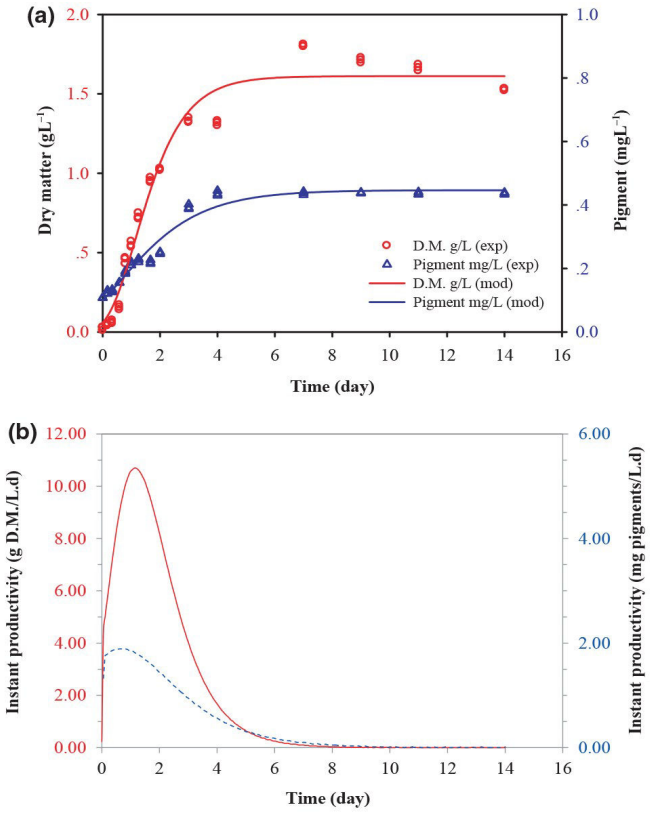

Fig. 5. Growth and pigment production by *Arthrobacter arilaitensis* Po102 cultivated in milk ingredient-based liquid medium. (a) Curves of dry matter and pigment production based on the experimental data and calculated using Gompertz model (○ = dry matter g L^{-1} from experimental data, — = dry matter g L^{-1} from calculating, ○ = pigment mg L^{-1} from experimental data, — = pigment mg L^{-1} from calculating). (b) Instant productivity of biomass and pigment calculated from the model curves (— = g dry matter L^{-1} day^{-1}, ---- = mg pigments L^{-1} day^{-1}).

Discussion

For a long time, the coloration of smear-ripened cheeses was quite entirely attributed to *B. linens* species. Then, many new dairy bacterial species were revealed and characterized. *Arthrobacter arilaitensis* has been recently putatively regarded as one important microorganism responsible for color development at the surface of the cheeses, resulting in a present lack of information and new experiments are needed to provide a better understanding of the role of this bacterium in cheese's coloration. Up to now, no experiment, as far as we know, had ever been conducted to examine the occurrence of pigment synthesis by *A. arilaitensis*, even though few previous studies investigated pigmented extracts prepared from yellow bacteria found on smear-ripened cheeses (Galaup *et al.*, 2005, 2007).

One among these previous studies seems close to the present experiment because the authors investigated the pigment extracts prepared from diverse unknown yellow bacteria isolated from smear-ripened cheeses using spectrophotometry and HPLC analysis (Galaup *et al.*, 2005). The absorption spectra of these yellow bacterial extracts

displayed characteristic of carotenoid pigments with three maxima wavelengths, 413.8, 437.9, and 466.8 nm. Continually devoting to the coloration of this kind of cheeses, Galaup *et al.* (2007) determined the pigment extracts from the rind of Maroilles, Mont d'Or and Epoisses. The results showed that the extracts consisted of some compounds presenting characteristic absorption spectra of carotenoids; additionally, some of these spectra were identical to these of the first previous study. Pigment compounds found in the extracts from both sources, bacterial cells and cheese rinds, were believed to be related to yellow bacteria, such as *Microbacterium gubbeenense* or *Arthrobacter arilaitensis*, based on HPLC profiles of extracts of bacteria strains isolated from the rind of smear-ripened cheeses; unfortunately, these bacteria had not been further taxonomically identified. However, chromatographic profiles as well as absorption spectra of the extracts prepared from eight *A. arilaitensis* strains used in the present study were similar to the previous ones, specially peak 1 (with three maxima: 413.8, 437.2, 465.3), suggesting that they might correspond to isomers of the same molecules. As a result of the retention times, the five peaks from the *A. arilaitensis* extracts represented five different compounds varying by their polarity level. C50 carotenoid decaprenoxanthin and its (*E*/*Z*) isomers and/or glycosides are expected yellow pigments produced by *A. arilaitensis* according to the genomic data previously published about strain Re117, which demonstrated the presence of a decaprenoxanthin-like carotenoid biosynthesis gene cluster (Monnet *et al.*, 2010). Respecting the spectrophotometric and HPLC analysis results, 14 strains of *A. arilaitensis* used in this study could be separated into two groups, carotenoid-producing and nonpigmented strains (Table S1). *Arthrobacter arilaitensis* strains in each group may have different responsibilities on cheese products; for example, the carotenoid-producing strains produce pigments resulting in the coloration of cheeses and flavors while the nonpigmented strains may be responsible for cheese flavor only. Deetae *et al.* (2007) described *A. arilaitensis* Mu107 as a strain involved in the flavor formation through the production of volatile aroma compounds such as sulfur compounds, aldehydes, and ketones.

The diversity of carotenoid production among eight *A. arilaitensis* strains (AA012, AA015, AA020, AA021, AA028, AA030, AA035, and CNRZ923) was low when cultivating under both conditions, light and dark. However, light was found to strongly affect carotenoid synthesis by all of these strains. In nonphotosynthetic bacteria, carotenoids play an important role as protectants against photo-oxidative damages (Liu *et al.*, 2012). Carotenogenesis in these microorganisms may occur in three manners including constitutive, light-induced, or cryptic manner. Despite the majority of microorganisms have been reported to synthesize carotenoids constitutively, some of them produce these pigments when the cells are illuminated (i.e. light-induced) as well as some other organisms form them in various manner (Takano *et al.*, 2005).

Kinetic of *A. arilaitensis* AA015 growth was not similar to the growth of *A. arilaitensis* 3M03 as well as the observation of *A. arilaitensis* Re117 from previous studies (Mounier *et al.*, 2008; Monnet *et al.*, 2010). The lag and stationary phases of *A. arilaitensis* AA015 were 14 h and 14–48 h, respectively, whereas *A. arilaitensis* 3M03 and Re117 needed roughly 6–7 days for the lag phase and 7–8 days for the exponential phase. Besides, the time used for these observations indicated that three strains including *A. arilaitensis* AA015, 3M03, and Re117 could run for a long time, as living *Arthrobacter* bacteria are able to survive over 30 days, even months. Nutrients and conditions used for cultivation are significant factors in bacterial growth, particularly iron which was reported to be an essential element for growth of *A. arilaitensis* (Monnet *et al.*, 2010, 2012). Consequently, a difference of nutrients content in the individual cultures probably affected growth rate, cell number, and pigment production of these *A. arilaitensis* strains.

A growth-associated pigmentation was applied here to explain the pigment production rate in *A. arilaitensis* as well as this is the case in a number of other carotenogenic microorganisms. Maximum pigment synthesis could also arise at the end of the exponential phase of growth or even during stationary phase, classifying biosynthesized carotenoids as secondary metabolites (Garcia *et al.*, 2005; Moliné *et al.*, 2009). In *A. arilaitensis* AA015, carotenoid pigments produced as primary metabolites would act as photo-oxidative protectants to prevent cells from damages.

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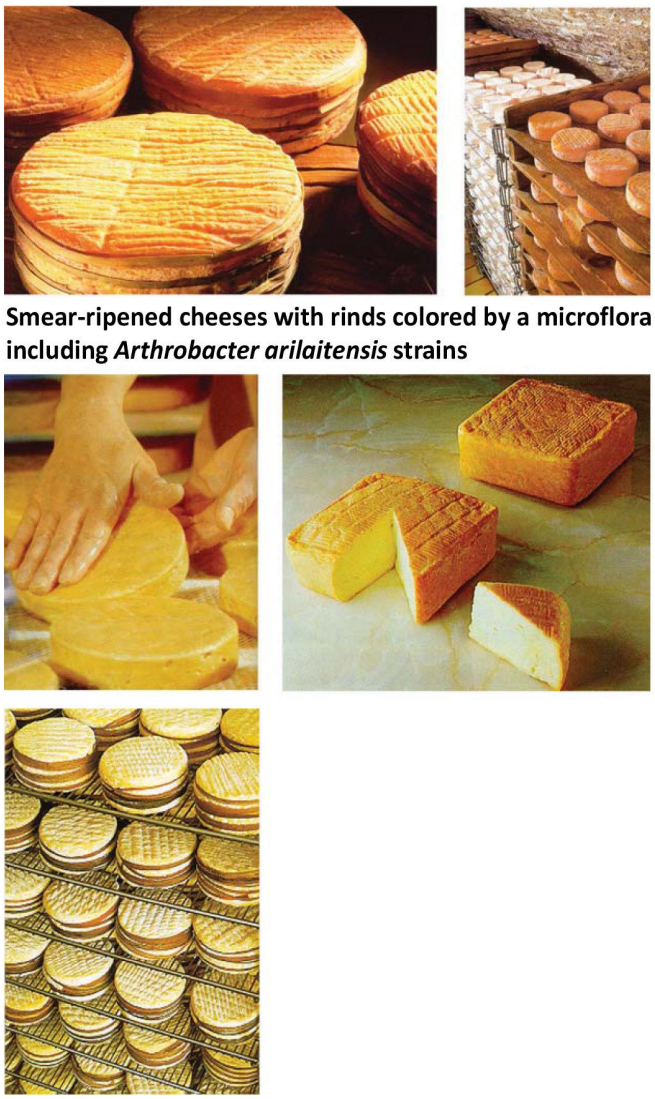
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Classification of the 14 *Arthrobacter arilaitensis* strains by the ability of carotenoid production under the condition and culture studied.

Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main article.



Smear-ripened cheeses with rinds colored by a microflora including *Arthrobacter arilaitensis* strains

Surface bacteria contribute to the overall color of smear-ripened cheeses and to consumer's purchasing decision. Yellow carotenoids production is described for the new player *Arthrobacter arilaitensis* depending on strains, culture media, light exposure, and growth kinetics.

Article 6

☒ C50 carotenoids from the cheese-ripening bacteria *Arthrobacter arilaitensis*. **Sutthiwong N.**, Giuffrida D., Dufossé L.. (En vue de soumettre à la revue internationale).

Pendant longtemps, *Brevibacterium linens* a été considéré comme le micro-organisme majeur, quasi unique, responsable du développement de la couleur à la surface des fromages en raison de sa capacité à produire des caroténoïdes orange qui ont été identifiés comme isorenieratene, 3-hydroxy-isorenieratene et 3,3'-di-hydroxy-isorenieratene. Toutefois, au cours des dernières années, d'autres micro-organismes ont été signalés comme aussi importants que *B. linens* pour la maturation. *Arthrobacter arilaitensis* a été récemment isolé sur des fromages à croûte lavée et elle a également été décrite comme l'un des principaux micro-organismes impliqués dans la pigmentation jaune des fromages de cette famille.

Nous avons étudié précédemment la présence de pigments synthétisés par une variété de souches de *A. arilaitensis* dans plusieurs aspects, dont les spectres d'absorption et de chromatogrammes HPLC des extraits pigmentaires. Sur la base des spectres d'absorption et des profils HPLC, les extraits méthanoliques préparés à partir des souches pigmentées en jaune contiennent au moins 4 caroténoïdes représentés principalement par des molécules polaires. Les caroténoïdes de la famille C50, décaprénoxanthine et ses (*E* / *Z*) et / ou glycosides, sont attendus comme étant les pigments jaunes générés par *A. arilaitensis* d'après les données génomiques publiées antérieurement, article qui a démontré la présence d'un groupe de gènes de biosynthèse des caroténoïdes comme la décaprénoxanthine.

Le but de cette étude est d'identifier les pigments synthétisés par les bactéries d'affinage de fromage *A. arilaitensis* afin de fournir des informations aussi complètes que possible pour expliquer la coloration des fromages à croûte lavée lors de l'utilisation de cette bactérie dans le cadre de la flore de maturation.

C50 carotenoids from the cheese-ripening bacteria *Arthrobacter arilaitensis*

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Abstract

Aim: To identify the pigments produced by the cheese-ripening bacteria *Arthrobacter arilaitensis*.

Methods and results: *Arthrobacter arilaitensis*, strain Po102 and strain Stp101, were used in this study. Pigments were extracted with methanol and methyl *tert*-butyl ether. An HPLC-PDA-APCI-MS methodology was applied to characterize the pigment profile. Based on the UV-Vis spectra, mass spectra, both in APCI (-) and APCI (+) ionization modes, and the elution order, eight different carotenoids were identified in both strains. In particular, four of them, namely, decaprenoxanthin, sarcinaxanthin, 9-*Z*-decaprenoxanthin and 15-*Z*-decaprenoxanthin, which were present as free main carotenoids, have been characterized by both their UV-Vis spectra and MS spectra both in APCI (-) and APCI (+) ionization modes. The other minor carotenoids, namely, sarcinaxanthin mono-glucoside pentaacetate, decaprenoxanthin mono-glucoside, decaprenoxanthin di-glucoside, decaprenoxanthin-C16:0 (decaprenoxanthin-palmitate), which were present in very low amounts, were only detected in the APCI (-) ionization mode.

Conclusions: Both strains of *A. arilaitensis* investigated appear to synthesize eight different C_{50} carotenoids; free all-*E*-decaprenoxanthin was the most abundant one.

Significance and Impact of the study: Our results provide the first chemical characterization of the pigments produced by the cheese-ripening bacteria *A. arilaitensis*, which had not previously been reported.

Keywords:

carotenoid, decaprenoxanthin, *Arthrobacter arilaitensis*, smear-ripened cheese, ripening bacteria

Introduction

Smear-ripened cheeses, also known as surface-ripened cheeses or red-smear ripened cheeses, are economically important dairy products, manufactured with the use of various microorganisms during the cheese-making process, mainly in milk coagulation and ripening stages. The surface of smear-ripened cheeses is a microbial mat, with a yellow-orange-reddish color, composed of a large diversity of bacteria and yeasts. Several smear-ripened cheeses are recognized by consumers through their color and form characteristics, for example, Livarot and Epoisses from France, Limburger and Tilsit from Germany, and Taleggio from Italy (Fig. 1). Therefore, surface color of smear-ripened cheese is defined to be one of the major attributes which impacts the consumer acceptance of these cheeses and which is also related to many cheese qualities, e.g. maturity, naturalness, flavorness and cleanliness (Dufossé et al. 2001). Coloration of smear-ripened cheeses appears to be a complex phenomenon due to the functional interactions among microorganisms on cheese rind (Leclercq-Perlat et al. 2004; Mounier et al. 2006; Mounier et al. 2008). Pigments generated by the smear bacteria at the surface of cheese were poorly studied, exception made for those of the bacteria *Brevibacterium linens*. For a long time, this bacterium was considered to be the single major microorganism responsible for the color development at the surface of cheeses because of its ability to produce orange carotenoids which were identified as isorenieratene, 3-hydroxy-isorenieratene and 3,3'-di-hydroxy-isorenieratene (Kohl et al. 1983) . However, during the last years, with the more accurate methods for taxonomic identification e.g. PCR-denaturing gradient gel electrophoresis and 16S sequencing, or total microbiome sequencing, other microorganisms have been reported as important as *B. linens* for the ripening of smear cheeses (Eliskases-Lechner and Ginzinger 1995; Bockelmann and Hoppe-Seyler 2001; Goerges et al. 2008) .

Arthrobacter bacteria have long been isolated from smear-ripened cheeses and they have also been notified as one of the major microorganisms involved in the surface pigmentation of the smear-ripened cheeses due to the characteristic overall color of the biomass and their occurrence at the different stages of the cheese production until the end of cheese ripening (Feurer et al. 2004; Mounier et al. 2005; Irlinger and Mounier 2009; Larpin-Laborde et al. 2011). *Arthrobacter arilaitensis* is one species among *Arthrobacter* bacteria present on smear-ripened cheeses, and was reported to be a dominant bacterial species on the cheese surface, showing the capacity of yellow pigment production (Galaup et al. 2005; Irlinger et al. 2005; Galaup et al. 2007; Mounier et al. 2008; Leclercq-Perlat and Spinnler 2010; Sutthiwong et al. 2014). Also in the genus *Arthrobacter*, the psychrophilic bacterium *Arthrobacter glacialis* was previously reported to synthesize three C_{50} carotenoids i.e. decaprenoxanthin, bisanhydrobacterioruberin and *A.g.* 470 (Arpin et al. 1975). *Arthrobacter* sp. M3 was also found to produce decaprenoxanthin and its glucosides (Arpin et al. 1972). In nature, C_{50} carotenoids are synthesized by bacteria which belong to the *Actinomycetales* order; until now, only three different C_{50}

Figure 1 Smear-ripened cheeses, popular dairy products made with a complex microflora at cheese surface: (A) a variety of smear-ripened cheeses; (B) Livarot; (C) Limberger; and (D) Taleggio.

carotenoid biosynthetic pathways have been described: *(i)* the β-cyclic C_{50} carotenoid C.p. 450 in *Dietzia* sp CQ4; *(ii)* the γ-cyclic C_{50} carotenoid sarcinaxanthin in *Micrococcus luteus* NCTC2665; and *(iii)* the ε-cyclic C_{50} carotenoid decaprenoxanthin in *Corynebacterium glutamicum* (Krubasik et al. 2001; Tao et al. 2007; Netzer et al. 2010; Heider et al. 2014). According to the whole genomic data previously published for *Arthrobacter arilaitensis* (Monnet et al. 2010), a decaprenoxanthin-like carotenoid biosynthetic gene cluster is present.

The aim of this study was to fully identify all the pigments biosynthesized by the cheese-ripening bacteria *A. arilaitensis,* in order to provide new information useful both to elucidate the nature of the smear-ripened cheeses color, and relevant when selecting this bacterium as a part of ripening flora.

Materials and methods

Bacterial strains, medium and growth conditions

Yellow-pigmented *Arthrobacter arilaitensis* strains isolated from smear-ripened cheeses, namely

A. arilaitensis Po102 from Pont-l'Évêque and *A. arilaitensis* Stp102 from Saint-Paulin, were used in this study. Usually stored at -80°C, they were maintained during this study on milk ingredient-based agar, stored at 4°C and subcultured monthly. The choice of these two strains was due to their strong yellow color among the whole collection of *Arthrobacter arilaitensis* strains of our laboratory. It was previously shown that all the strains presented the same HPLC pigment profile (Sutthiwong & Dufossé, 2014), including the Re117 strain used by Monnet et al. (2010) for the whole genome sequencing.

Starting from a loop of pure bacteria, the *A. arilaitensis* strains were primarily prepared as 72-h-old precultures in milk ingredient-based liquid medium (0.5% Difco casamino acids, 0.1% Bacto yeast extract, 2% glucose, 0.5% NaCl, 0.1% KH_2PO_4, adjusted pH to 7.0 ± 0.2) at 25 °C with 150 rpm agitation using INFORS HT incubator. After 72 h, 1% (v/v) of each preculture was inoculated in the milk ingredient-based liquid medium, and then incubated in the same conditions as the preparation of preculture.

Pigment extraction

A. arilaitensis cultures grown under aerobic conditions with continuous shaking were harvested after 7 days by centrifugation at 6000 *g* for 15 min. After washing twice with deionized water, the cell pellets were frozen at -80°C for 48 h, and then lyophilized to complete dryness (about 3 days). One g of lyophilized cells was extracted with 99% methanol (Carlo Erba) by sonication, at room temperature for 90 min. Centrifugation (6000 *g*) for 15 min was carried out to separate the cell debris and the carotenoid containing supernatant. The cell debris were re-extracted 2 times with methanol and 2 times with methyl *tert*-butyl ether (MTBE, Carlo Erba) until complete bleaching of the biomass was obtained. Total volume of the combined extracts was evaporated to dryness under vacuum at 55 °C using a Büchi Rotavapor. The residue was dissolved in 1 ml methanol/MTBE (1:1 v/v), filtered through Millex-GV 0.2 -µm hydrophilic membrane (Millipore), and stored under -20 °C in an amber vial prior to HPLC analysis.

HPLC-PDA-APCI-MS analysis

Analyses were carried out on a Nexera liquid chromatography system (Shimadzu, Milan, Italy), consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A_5 degasser, a CTO-30A column oven, a SIL-30A autosampler. 0.1 mm I.D. stainless steel tubing (zero dead volume) was employed for column connection. The LC system was coupled to an LCMS-IT-TOF mass spectrometer through an APCI source operating in both positive and negative mode(Shimadzu, Kyoto, Japan). Data acquisition was performed by means of the LCMS solution software (Version 3.50.346, Shimadzu).

Chromatographic separation was achieved on Ascentis Express Fused-core C18 columns, 150x4.6 mm i.d., 2.7 µm d.p., kindly donated by Supelco/Sigma-Aldrich (Bellefonte, PA, USA). The mobile phases consisted of methanol (eluent A) and methyl *tert*-butyl ether (eluent B). Elution from the column was isocratically carried out from 100% (A) for 25 min then a gradient was established over the next 45 min with (B) to a final ratio of 70:30 (A:B), followed by a further elution for 15 min with 70:30 (A:B). The column was then returned to the initial conditions and equilibrated over 20 min. The flow rate was 1 ml/min and the injection volume was 20 µL. The UV-Vis spectra were acquired in the range of 250-600 nm, while the chromatograms were extracted at 440 nm.

LCMS-IT-TOF MS detection parameters were as follows: detector voltage, 1.50 kV; interface temperature: 400 °C; CDL temperature, 250 °C; block heater temperature, 230 °C; nebulizing gas flow (N_2), 2.5 L/min; ion accumulation time, 30 msec; full scan range, 300-800 *m/z*; event time, 300 ms; repeat, 3; ASC, 70%. For MS/MS, full scan range, 50-800 *m/z;* ion accumulation time, 30 msec; CID energy: 50%. Samples were analyzed in triplicate.

Carotenoids were tentatively identified by their of UV-Vis spectra, including spectral fine structure, and MS spectra recorded in both positive and negative APCI ionization modes, and also considering their respective elution order.

Results

The carotenoid extracts obtained from the smear-ripening bacteria *A. arilaitensis*, strains Po102 and Stp101, presented similar chromatographic profiles as we previously observed for a larger number of strains (Sutthiwong & Dufossé, 2014). A typical chromatogram (Fig. 2) recorded at 440 nm, displayed 2 major groups of peaks according to the elution time. Group I contained peak 1, eluting at 9.79 min, followed roughly 1 min later by a small but marked peak 2. Following group I, peak 3 and peak 4 representing the group II were later eluted, between 16.5 and 17.5 min.

The main carotenoids were identified as decaprenoxanthin (1), sarcinaxanthin (2), 9-*Z*-decaprenoxanthin (3) and 15-*Z*-decaprenoxanthin (4). These molecules have clearly been characterized by both their UV-Vis spectra and MS spectra, both in APCI (-) and (+) ionization modes. Figure 2 also shows the PDA UV-vis spectra of the identified peaks.

The mass spectra of the two principal types of carotenoids biosynthesized by *A. arilaitensis* i.e. decaprenoxanthin (1) and sarcinaxanthin (2) in APCI (-) and APCI (+) ionization modes are shown in Fig 3.

The mass spectra clearly show the pseudomolecular radical ion at *m/z* 704 in the negative ionization mode for decaprenoxanthin (A) and sarcinaxanthin (C) and in the respective positive

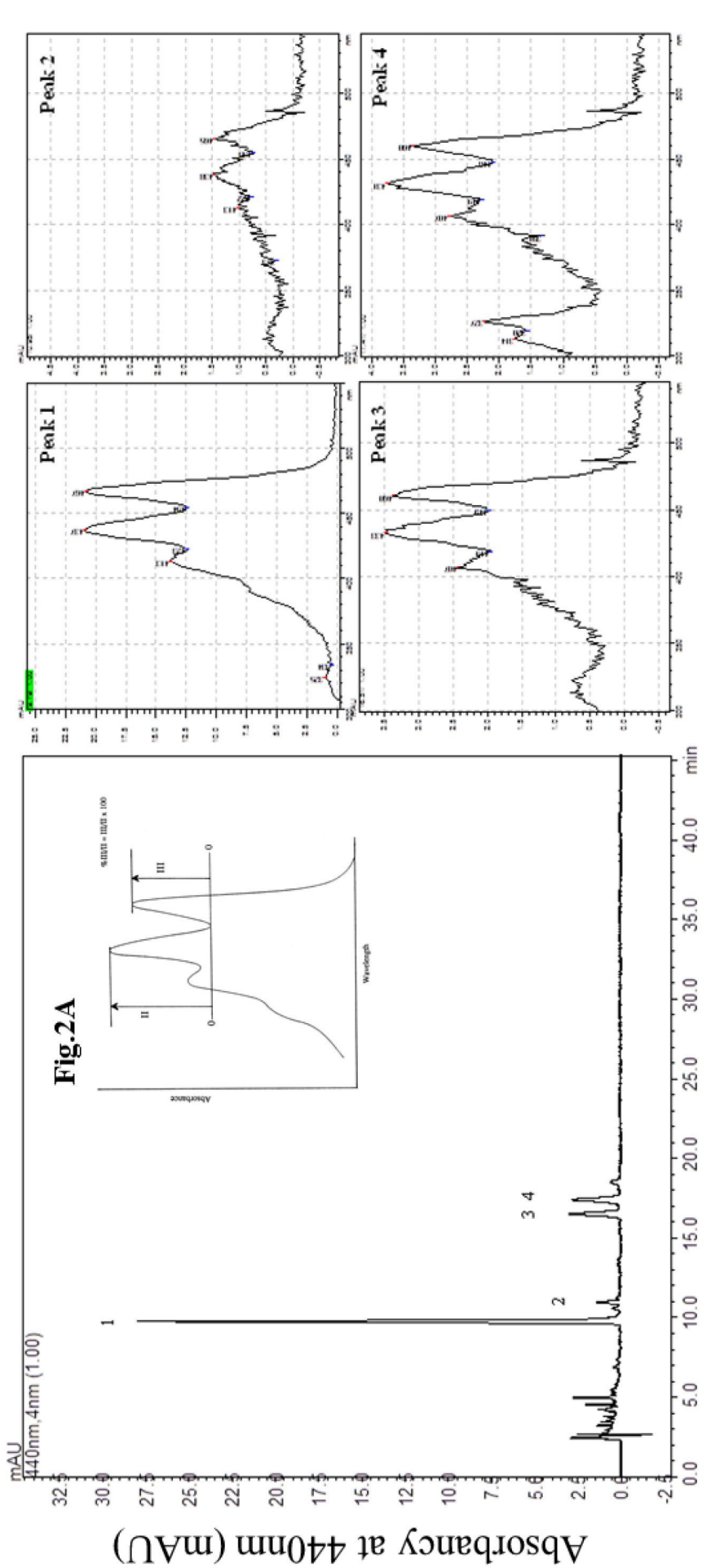

Figure 2 HPLC chromatographic profile at 440 nm of carotenoid extract from *Arthrobacter arilaitensis* Po102, and the respective PDA UV-vis spectra for peaks number 1, 2, 3, and 4. % composition of the main peaks from the PDA data are: Peak 1, 87.0%; Peak 2, 3.5%; Peak 3, 4.5% and Peak 4, 4.5%. As a way to analyze the fine structure of UV-vis spectrum of carotenoids, the base-line or zero value is taken as the minimum between the two peaks, the peak height of the longest-wavelength absorption band is designated as III, that of the middle absorption band (usually λ_{max}) as II (Fig. 2A) (Britton, 1995).

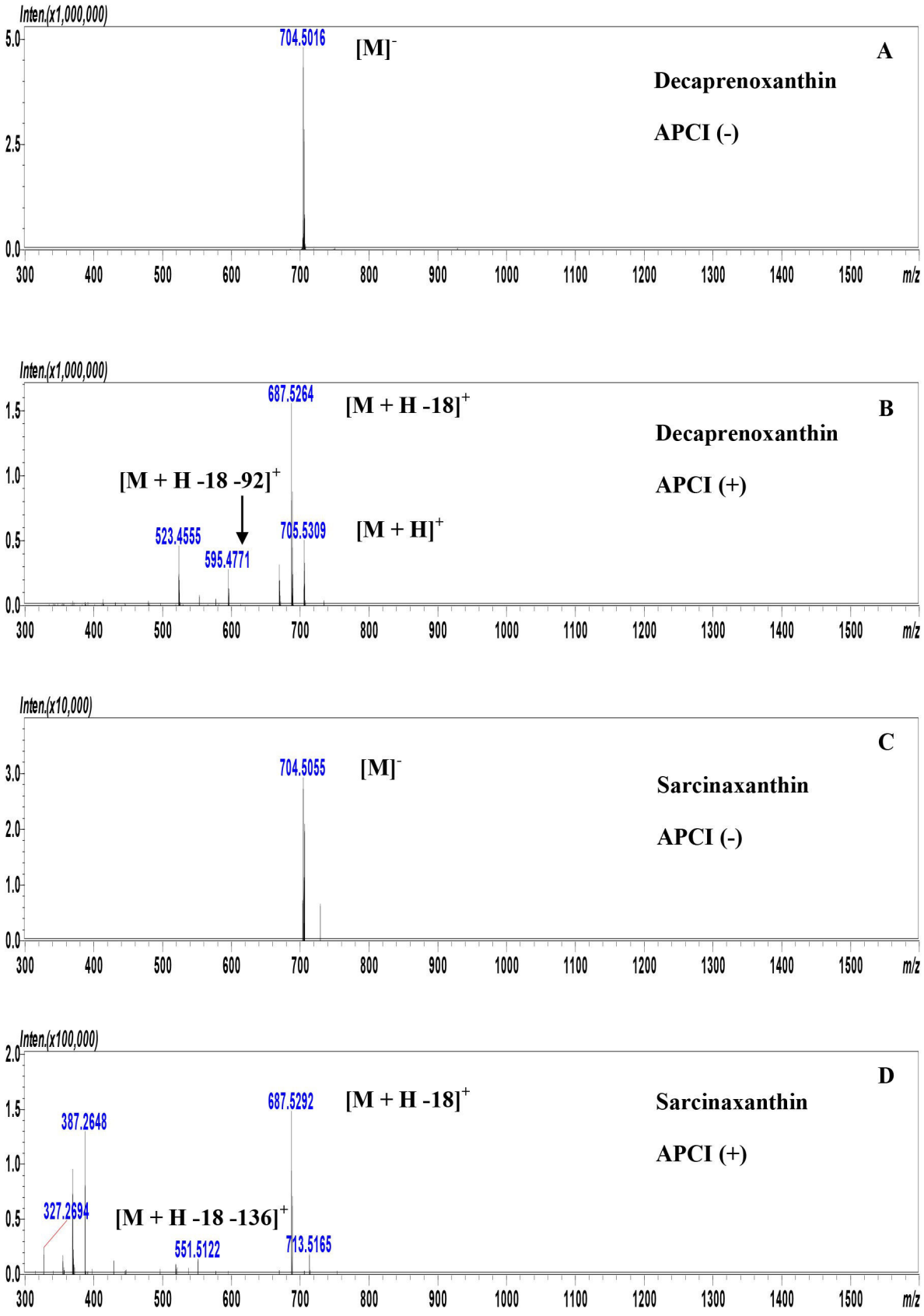

Figure 3 The mass spectra of decaprenoxanthin and sarcinaxanthin both in APCI (-) and APCI (+) ionization modes. (A) decaprenoxanthin in APCI negative mode, (B) decaprenoxanthin in APCI positive mode, (C) sarcinaxanthin in APCI negative mode, (D) sarcinaxanthin in APCI positive mode.

ionization modes (B and D), the fragments ions produced in the APCI source, by the loss of water, and toluene from decaprenoxanthin (B), and by the loss of water and the fragment at *m/z* 136, from sarcinaxanthin (D).

The other minor carotenoids which were present in very low amount, were only detected in APCI (-) ionization mode. These carotenoids were identified as sarcinaxanthin mono-glucoside pentaacetate (SMGp), decaprenoxanthin di-glucoside (DDG), decaprenoxanthin mono-glucoside (DMG) and decaprenoxanthin-C16:0 (decaprenoxanthin–palmitate, Dec-C16:0). Fig 4 shows the extracted ions chromatograms at *m/z* 849 (1028-179, for DDG), at *m/z* 866 for DMG, at *m/z* 942 for Dec-C16:0, and at *m/z* 704 relative to compound 1, 2, 3 , 4 as reported in Table 1, together with the UV-Vis and MS spectra information for the identified compounds.

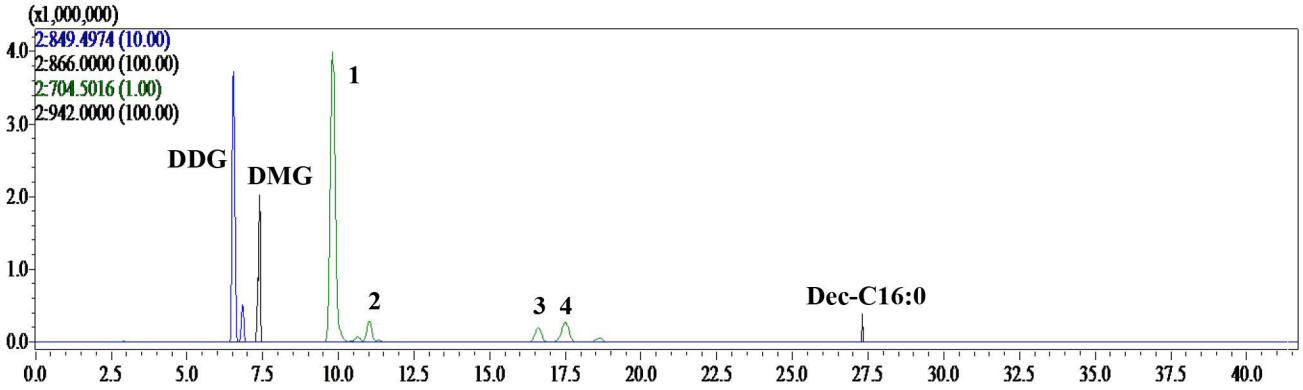

Figure 4 Extracted ions chromatogram (APCI negative) at *m/z* 866, *m/z* 849 (1028-179), *m/z* 942, and *m/z* 704, of the carotenoid extract of *Arthrobacter arilaitensis* Po102.

Table 1 Data obtained for UV-Vis, MS APCI (-) and MS APCI (+). Information and tentative identification of the carotenoid extract of *Arthrobacter arilaitensis* Po102.

ID	Identification	UV-Vis maxima	MS data APCI (-)	MS Data APCI (+)
SMG p	Sarcinaxanthin mono-glucoside pentaacetate	Not detected	1076	Not deteted
DDG	Decaprenoxanthin di-glucoside	Not detected	1028, 849	Not detected
DMG	Decaprenoxanthin mono-glucoside	Not detected	866	Not detected
1	Decaprenoxanthin	413, 437, 467	704	705, 689, 687, 669, 595
2	Sarcinaxanthin	415, 438, 466	704	705, 687, 551
3	9-Z-decaprenoxanthin	318, 409, 434, 460	704	705, 687, 669, 549, 523
4	15-Z-decaprenoxanthin	320, 407, 431, 460	704	687, 669, 577
Dec-C16:0	Decaprenoxanthin-C16:0	Not detected	942	Not detected

The chemical structures of the two most important carotenoids, from a metabolic point of view, produced by *A. arilaitensis*: *(i)* decaprenoxanthin, (2*R*,6*R*,2'*R*,6'*R*)-2,2'-Bis-(4-hydroxy-3-methylbut-2-enyl)-ε,ε-carotene; and *(ii)* sarcinaxanthin, (2*R*,6*R*,2'*R*,6'*R*)-2,2'-Bis-(4-hydroxy-3-methylbut-2-enyl)-γ,γ-carotene, are shown in Fig. 5. The whole picture of the eight carotenoids detected in our research is also presented in this framework of a biosynthetic pathway.

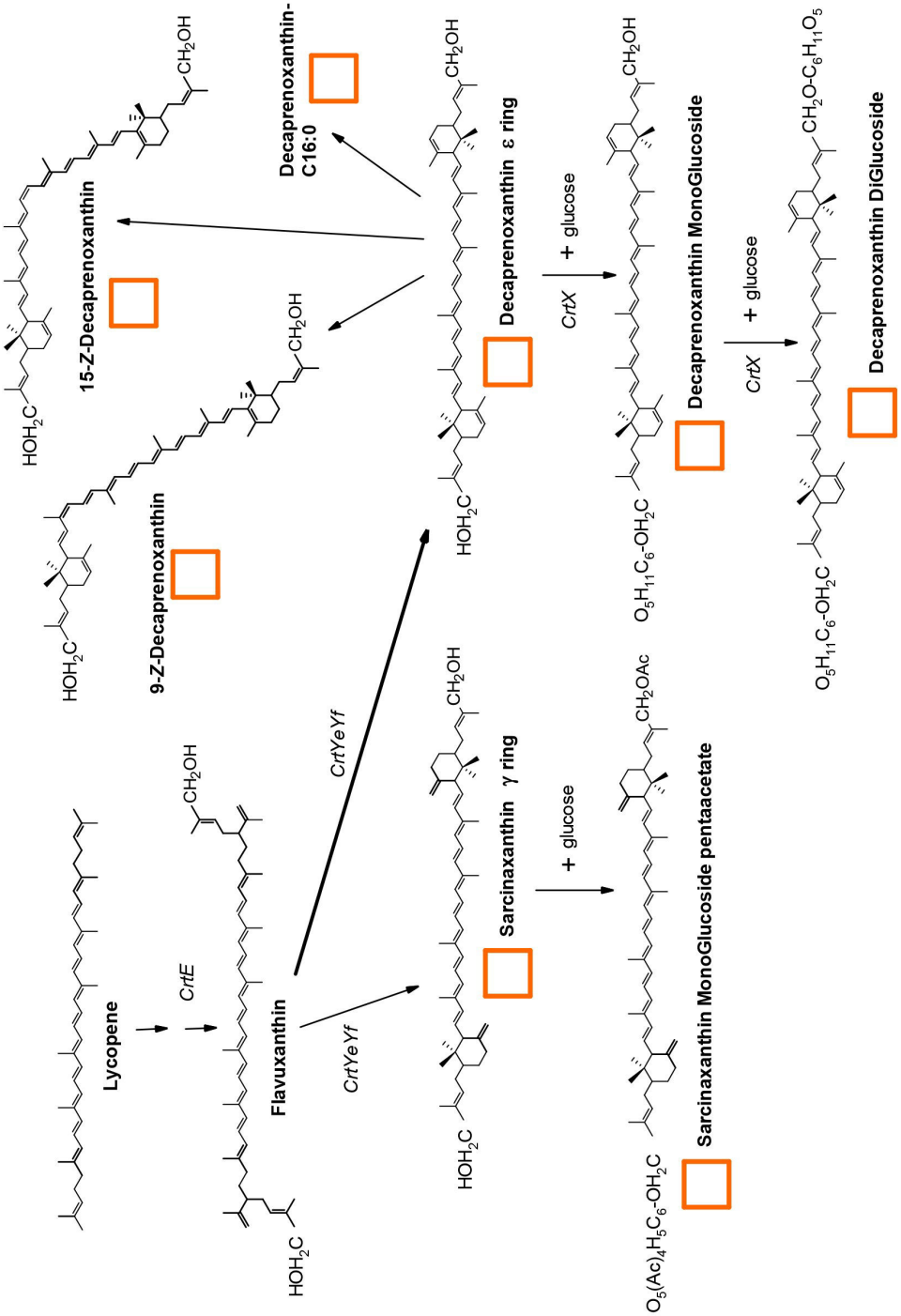

Figure 5 Part of the biosynthetic pathway of C50 carotenoids in *Arthrobacter arilaitensis*. Chemical structures of *(i)* decaprenoxanthin (2*R*,6*R*,2'*R*,6'*R*)-2,2'-Bis-(4-hydroxy-3-methylbut-2-enyl)-ε,ε-carotene; and *(ii)* sarcinaxanthin (2*R*,6*R*,2'*R*,6'*R*)-2,2'-Bis-(4-hydroxy-3-methylbut-2-enyl)-γ,γ-carotene

Discussion

In this study, the carotenoid extracts from the cheese-ripening bacteria *A. arilaitensis* strains were characterized by an HPLC-PDA-APCI-MS methodology. According to the data of the UV-Vis spectra, the mass spectra both in APCI (-) and APCI (+) ionization modes, and the elution order, *A. arilaitensis* species does indeed produce eight C_{50} carotenoids, and in particular, it mainly produced free all-*E*-decaprenoxanthin (carotenoid with ε ring), and in minor amount two of it *cis* isomers, the 9-*Z*-decaprenoxanthin and 15-*Z*-decaprenoxanthin. Decaprenoxantin is also present as *i)* mono- and di-glucosides and *ii)* ester with palmitic acid as acid moiety.

9-*Z*-decaprenoxanthin and 15-*Z*-decaprenoxanthin isomers are always in the same proportions within these bacterial pigmented extracts, analyzed in our lab for many years (over 100 injections) (before the official description of the new species *A. arilaitensis* in the literature or the in-deep analysis of the chemical structures of the pigments in the present study). These are not artefacts as many previous authors concluded that *Z* isomers of carotenoids occur naturally (Melendez-Martinez, Stinco, Liu and Wang, 2013).

The second interesting point in our study is the concomitant presence of sarcinaxanthin (carotenoid with γ ring) and a sarcinaxanthin derivative (sarcinaxanthin monoglucoside pentaacetate). To our knowledge, this is the first time that the simultaneous presence of these two C50 cycles is described in a non-genetically engineered bacteria. Up to now sarcinaxanthin biosynthesis was described in *Micrococcus luteus* (Netzer et al., 2010) and decaprenoxanthin biosynthesis in *Corynebacterium glutamicum* (Krubasik et al. 2001; Heider et al. 2012).

Genomic data previously published about *A. arilaitensis* Re117 (whole genome sequencing) described an *Idi, CrtE, CrtB, CrtI, CrtEb, CrtYe and CrtYf* carotenogenesis cluster, similar to the cluster of the decaprenoxanthin producing *Corynebacterium glutamicum* (Krubasik et al. 2001; Monnet et al. 2010; Heider et al. 2012). Cyclases are key enzymes in the biosynthesis of C50 carotenoids and were investigated through molecular biology. The *M. luteus CrtYgYh* polypeptides constitute a γ-cyclase specifically converting flavuxanthin into sarcinaxanthin. In contrast, the *C. glutamicum CrtYeYf* polypeptides constitute both γ-cyclase and ε-cyclase activities and can convert flavuxanthin into three different C50 carotenoids; decaprenoxanthin, sarcinaxanthin, and sarprenoxanthin. For *A. arilaitensis,* only the first two carotenoids were detected.

Since only a few studies have been dedicated to *A. arilaitensis*, the pigments of this bacterium had never been investigated further than through its occurrence on the smear-ripened cheeses (Galaup et al. 2007). To our knowledge, the present results were therefore the first accurate identification of these carotenoid pigments produced by *A. arilaitensis*.

Carotenoids are a group of yellow to orange-red terpenoid pigments synthesized by a large variety of plants, algae and microorganisms. These pigments have been detailed as possessing several important functional properties, principally antioxidant activity, as well as prevention of certain diseases e.g. cancer, cardiovascular and Alzheimer's pathologies (Kirsh et al. 2006; Li et al. 2012). By the beneficial effects of natural functions of carotenoids, they have been increasingly applied in a wide range of food products, pharmaceutics and cosmetics, as well as employed in the animal feed industry for livestock, poultry, fish and crustaceans (Wang et al. 2006; Yuan et al. 2011; Anunciato and Fiho 2012; Pickworth et al. 2012; Tarique et al. 2013)

More than 95% of all natural carotenoids are based on a symmetric C40 phytoene backbone, and only a small number of C30 and even fewer C50 carotenoids have been discovered in Nature. C50 carotenoids have multiple conjugated double bonds, and they contain at least one hydroxyl group; both these features contribute to strong antioxidative properties. Therefore, the C50 carotenoids, not produced by plants, have high potential for application in nutraceuticals and pharmaceuticals.

Besides the conventional use in cheese manufacturing, the new information about carotenoids synthesized by the cheese-ripening bacteria *A. arilaitensis* may afterward promote the acceptability for ingredients extracted from food-grade bacteria which would lead to the enlargement of the panel of carotenoids available as food colorants as well as for other purposes, e.g. the application of C_{50} carotenoids in light protecting cosmetics and sunscreens as these pigments were previously described to be effective UV and visible light filters.

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Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

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Article 7

☒ *Arthrobacter arilaitensis* strains isolated from ripened cheeses: characterization of their pigmentation using spectrocolorimetry. **Sutthiwong N**., Caro Y., Milhau C., Valla A., Fouillaud M., Dufossé L.. Food Research International, 2014, 65, 184-192.

La couleur est un élément clé de la décision d'achat d'un consommateur en raison de ce qu'elle apparaît comme un signe de qualité, y compris un aspect attrayant pour de nombreux produits. Dans les fromages, en particulier les fromages à pâte molle croûte lavée, la couleur est un signe potentiel de plusieurs qualités par exemple la maturité, le naturel et la saveur. De nombreux fromages à croûte lavée sont reconnus par un consommateur selon leur couleur caractéristique.

Depuis quelques décennies, la spectrocolorimétrie a été largement utilisée en association avec la technologie alimentaire, en particulier dans les sciences de la viande, ou pour l'obtention de l'uniformité de la couleur de différents types de produit. Dans le cas de la coloration du fromage, la mesure de la couleur à l'aide d'un spectrocolorimétrie a été appliquée pour évaluer le développement de la couleur à la surface du fromage en fonction du temps, pour le contrôle de la qualité, ainsi que comme un outil pour la caractérisation de la pigmentation de la microflore du fromage.

Dans le but de fournir des informations aux fabricants de fromage lors de l'usage d'*Arthrobacter arilaitensis* comme une partie de la flore d'affinage, cet article examine l'utilisation de la spectrocolorimétrie quantitative pour la description des pigmentations fournies par différentes souches de cette bactérie.

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Arthrobacter arilaitensis strains isolated from ripened cheeses: Characterization of their pigmentation using spectrocolorimetry

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ABSTRACT

Pigmentation of a total 14 strains of *Arthrobacter arilaitensis* isolated from smear-ripened cheeses was evaluated using quantitative spectrocolorimetry. After 14 days of cultivation on milk ingredient-based solid medium on Petri dish, the bacterial biofilms were measured and expressed in the CIE L^*a^*b colorimetric system. Alignments of hue value from each experimental (a^*b) pair ranged from 72.39 to 240.83° that could be statistically divided into 9 groups ($P < .05$). Eight strains, out of 14, presented yellow to greenish-yellow pigmented cultures. The effect of light exposure against storage in the dark was also investigated using this approach. Three groups depending on a coloration behavior affected by light were illustrated for these 14 *A. arilaitensis* strains e.g. positively sensitive, negatively sensitive and not sensitive to light. This study is the first one describing the color contribution of *A. arilaitensis*, an emerging industrial dairy bacterium, to cheese ripening.

1. Introduction

Red-smear ripened soft cheeses, characterized by their orange-red color on rind, are dairy products widely consumed in Europe. The color is due essentially to carotenoids, in combination with other pigments, produced by the cheese microflora during ripening (Fig. 1). The microbiology of these cheeses is complex and not entirely well understood yet, as it results in interactions between bacteria and yeast strains. For a long time, *Brevibacterium linens* was considered to be the major microorganism responsible for the color development at the surface of the cheeses because of its ability to produce orange carotenoids (Kohl, Achenbach, & Reichenbach, 1983). However, other bacteria have been recently reported as important as *B. linens* for the ripening of the cheese rind (Brennan et al., 2002; Eliskases-Lechner & Ginzinger, 1995; Feurer, Vallaeys, Corrieu, & Irlinger, 2004; Mounier et al., 2005; Valdès-Stauber, Scherer, & Seiler, 1997). Furthermore, it is believed that the coloration of cheese is a consequence of complex interactions between yellow-pigmented *Arthrobacter* sp. and other microorganisms (Bockelmann & Hoppe-Seyler, 2001; Galaup et al., 2007; Irlinger & Mounier, 2009). One important strain among the genus *Arthrobacter* found in ripened-cheeses is *Arthrobacter arilaitensis* (Feurer et al., 2004; Irlinger, Bimet, Delettre, Lefèvre, & Grimont, 2005; Mounier et al., 2005). The colonies of *A. arilaitensis* commonly exhibit yellow colors and these bacteria have been found at different stages of ripening. It is then assumed that *A. arilaitensis* could be one of the microorganisms that would produce pigments on cheese rind, contributing to its characteristic overall color (Feurer et al., 2004; Galaup et al., 2007; Larpin-Laborde et al., 2011).

The color is a key element of a consumer's purchasing decision due to its appearance as a sign of quality including an attractive aspect for numerous products. A quantitative spectrocolorimetry method is routinely applied for handling the consistency of color for various kinds of product. Indeed a spectrocolorimeter emulates the psychosensorial mechanism of the color perception and interpretation of human beings. It integrates both light source spectrum and the object color spectrum into the reflectance spectrum. The latter is further filtered in order that the red, green and blue spectra are extracted as three coordinates, imitating the response of the human eye to light and color. This information is then processed to provide the three dimension $L^*a^*b^*$ response (Hutchings, 1994).

For a few decades spectrocolorimetry has been broadly used in association with food technology, especially in meat science. Spectrocolorimetry in the CIE $L^*a^*b^*$ colorimetric system has been fully applied by Millar, Moss, McDougall, and Stevenson (1995) to

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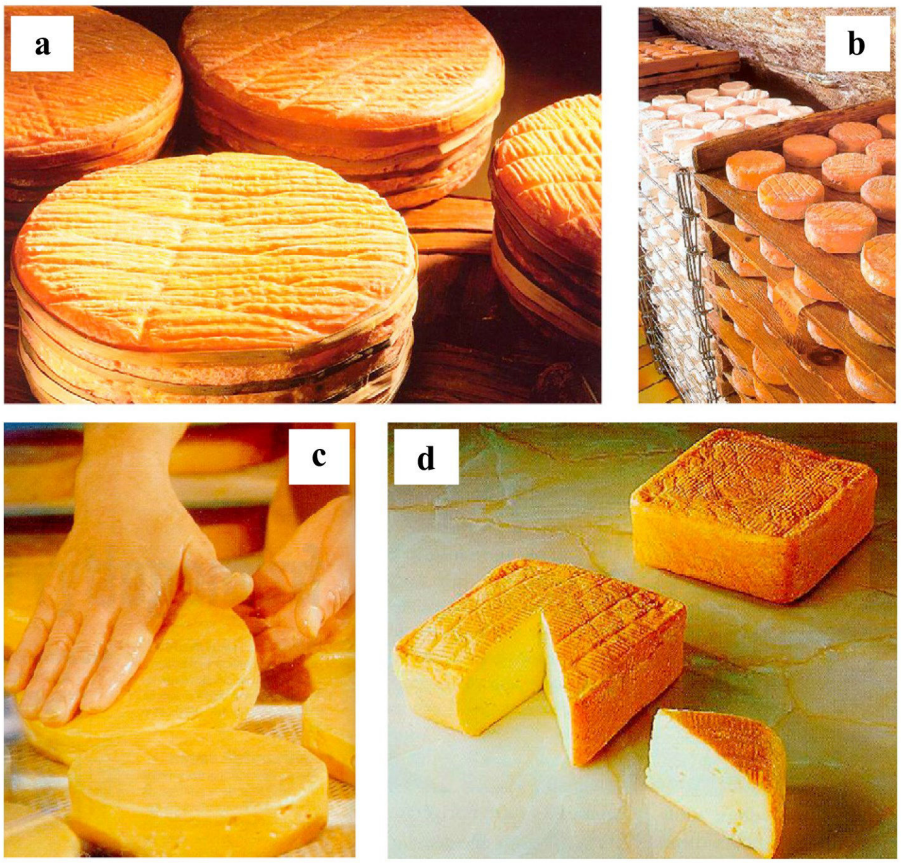

Fig. 1. Smear-cheeses with rinds colored by a microflora including *Arthrobacter arilaitensis* strains. a. Livarot, b. Rollot in the ripening cellar, c. Hand-made washing of Munster, d. Maroilles.

study ionized chicken meat and by Vorst, Clarke, Allison, and Booren (2004) to determine the effects of radio frequency transponders on beef muscle. Üren and Babayi it (1996) also used it to measure the color of Turkish-type fermented sausage. Furthermore, this apparatus has been used in fishery science to study the color of raw carp fish filet affected by high pressure processing at a low temperature (Sequeira-Munoz, Chevalier, Le Bail, Ramaswamy, & Simpson, 2006) as well as to investigate the effect of dietary astaxanthin on color of rainbow trout filet (Choubert, Blanc, & Vallée, 1997; Choubert, Mendes-Pinto, & Morais, 2006). Besides meat and fishery sciences, the color measurement using spectrocolorimetry was also used to evaluate the impact of short wave ultraviolet treatments on liquid eggs products (de Souza & Fernández, 2012).

In the case of cheese coloration, Dufossé, Galaup, Carlet, Flamin, and Valla (2005) investigated the rind's color of various 'Protected Designation of Origin — PDO' cheeses and also assessed color development at the surface of cheese versus time for quality control. Marchesini, Balzan, Segato, Novelli, and Andrighetto (2009) also evaluated the effect of ripening period on the color of Asiago cheese, and Ramírez-Navas and Rodríguez de Stouvenel (2012) collected the color data of Quesillo cheese and used them as a part of quality control in the manufacturing process. In addition, spectrocolorimetry has been used as a tool for characterizing pigmentation of cheese microflora (Dufossé et al., 2005; Guyomarc'h, Binet, & Dufossé, 2000). The pigmentation of 23 *B. linens* strains cultivated under 2 conditions, light and dark, was assessed. Numerous bacterial strains isolated from Munster cheese were also determined and their color coordinates were projected with $L^*a^*b^*$ system. Masoud and Jakobsen (2003) followed the influence of pH, NaCl and interactions with yeast strains on the intensity of pigmentation produced by four coryneform bacteria. Furthermore, this device has been used to evaluate the coloring capacity of smear-ripened strains when cultivated under various conditions e.g. types of cheese curds, coculture with yeast strains used for cheese deacidification, etc. (Leclercq-Perlat, Corrieu, & Spinnler, 2004; Leclercq-Perlat & Spinnler, 2010; Mounier et al., 2006).

For the purpose of providing information to cheese-manufacturers when selecting *A. arilaitensis* as a part of ripening flora, this study investigates the use of quantitative spectrocolorimetry for the description of the pigmentation provided by different strains from this species.

2. Materials and methods

2.1. Bacterial strains

Fourteen *A. arilaitensis* strains isolated from smear-ripened cheeses were used in this study (Table 1). The strains were isolated from cheeses in our lab or were kindly provided by the Institut National de la Recherche Agronomique (INRA), France. Usually stored at −80 °C, they were maintained during this study on milk ingredient-based agar, stored at 4 °C and subcultured monthly.

2.2. Cultivation media and conditions

2.2.1. Milk ingredient-based medium

The milk ingredient-based medium containing 5 g Casamino acids (Difco), 1 g yeast extract (BD Bacto), 5 g NaCl, 20 g D-glucose (Fisher Scientific), 1 g KH_2PO_4 (Fisher Scientific) and 15 g agar granulated (BD Difco) per liter of deionized water was prepared. Before sterilizing at 121 °C, 15 min, the pH of the medium was adjusted to 7.0 ± 0.2.

For the characterization of pigmentation, the culture medium was poured in 90 mm diameter Petri dishes. Each *A. arilaitensis* strain was suspended in peptone saline diluent [1 g casein peptone (Sigma) and 8.5 g NaCl (Fisher Scientific), adjusted pH to 7.0 ± 0.2 at 25 °C] and 1 ml of a 10^7 cell suspension was spread over the surface of the solid

Table 1
The 14 *Arthrobacter arilaitensis* strains used in this study.

Strain	Cheese origin
A. arilaitensis AA009	Livarot, France
A. arilaitensis AA012	Livarot, France
A. arilaitensis AA014	Munster, France
A. arilaitensis AA015	Pont-l'Evêque, France
A. arilaitensis AA017	Smear cheese, France
A. arilaitensis AA020	Munster, France
A. arilaitensis AA021	Epoisses, France
A. arilaitensis AA025	Livarot, France
A. arilaitensis AA028	Saint-Paulin, France
A. arilaitensis AA030	Reblochon, France
A. arilaitensis AA035	Livarot, France
A. arilaitensis AA036	Munster, France
A. arilaitensis CNRZ923	Smear cheese, Centre National de Recherches Zootechniques (CNRZ) strain collection, Paris, France
A. arilaitensis WS2230	Smear cheese, Weihenstephan (WS) strain collection, Freising, Germany

medium. All Petri-dishes were then incubated at room temperature for 14 days.

2.2.2. Light exposure

The trials were carried out in independent triplicates under two different conditions at room temperature, natural daylight and darkness. The plates corresponding to dark condition were wrapped in aluminum foil and incubated in a dark place.

2.3. Color measurements

The color of the milk ingredient-based agar inoculated with *A. arilaitensis* strains was measured using CM-3500d spectrocolorimeter (Minolta Co., Ltd., Japan) driven with SpectraMagic *NX* Pro. software (Minolta). The reference illuminant was D65 (standard daylight). According to the Compagnie Internationale de l'Eclairage (CIE, 1978) the data were reported in the $L^*a^*b^*$ colorimetric system. A single-pieced disk of agar, large enough to cover the entire light spot, was cut in each Petri dish, from an area of homogeneous colony development. The sample was then held culture-down and layered in the bottom of a 45 mm diameter CM-A128 glass Petri dish (Minolta) for incident light color measurements.

To characterize a color in the CIE $L^*a^*b^*$ color system, 3 colorimetric coordinates are obtained from the spectrocolorimeter. L^* defines lightness (ranges from 0% to 100%, dark to light), a^* indicates the red/green value (from −60 to 60, green to red), and b^* denotes the blue/yellow value (from −60 to 60, blue to yellow). The attributes of color, C^* and $h°$, describe the chroma (vividness or dullness) and the hue angle or tone of the color, respectively. The value of chroma C^* is 0 at the center and increases according to the distance from the center. Hue angle $h°$ is defined as starting at the $+a^*$ axis and is expressed in degrees: 0° would be $+a^*$ (red), 90° would be $+b^*$ (yellow), 180° would be $-a^*$ (green), and 270° would be $-b^*$ (blue). Hue values correspond to the angle of the a^*/b^* coordinate of the points.

$$\text{Chroma } C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$\text{Hue angle } h° = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

2.4. Statistical analysis

The data were analyzed using SigmaPlot software (Systat Software, Inc., USA). The *t*-test was performed for comparing mean values of individual variable of each strain between the 2 conditions at 95% significance level. When comparing data of each variable among 14 strains, one-way analysis of variance (one-way ANOVA) was applied. The difference of considered variable was estimated by Tukey HSD test according to a α risk of 5%.

3. Results and discussion

3.1. Diversity of A. arilaitensis strains pigmentations

Total 42 natural daylight experimental (a^*, b^*) pairs displayed in the CIE $L^*a^*b^*$ color space are shown in Fig. 2. Among the whole set of these 42 responses, the a^* values extended from −3.64 to 0.92, with b^* value ranging from −5.36 to 14.64. The hue varied between 72.39 and 240.83° that could be statistically divided into 9 groups. One among them is composed of 5 strains (AA012, AA015, AA021, AA030, and CNRZ923) showing close responses with hues ranging from 92.61 to 98.57° (yellow) (Fig. 3). Two additional minor groups which consisted in only a single or 2 strain(s) including AA020, AA028 and AA035 displayed yellow or greenish-yellow pigments, hue angles varying from 84.37 to 112.09. Besides these strains, $h°$ values of the strain AA014 (72.39) and the other 5 strains namely AA009, AA017, AA025, AA036 and WS2230 with hues varying from 118.52 to 240.83° were identified as cream or pale (poorly colored).

When inquired to identify the color of an object, however, people will most likely describe first its hue. Very simply, hue is how an observer perceives an object's color — yellow, orange, pink, green, blue, etc. The colorimetric circle in Fig. 2 shows the continuum of color from one hue to the next. As the circle illustrates, if two pigments colored yellow and green were mixed, a new colored pigment would show yellow-green. Add green to blue for green-blue, and so on.

As the dimension of data plots projected onto a (a^*, b^*) plane was scattered and did not completely match one ray (hue) of the scale, a correlation between the two chromatic coordinates, a^* and b^*, was not suitable to explain the whole data. This evidence indicated that the ratio of pure red ($+a^*$) did not depend on the yellow component ($+b^*$), which led to a unique occurrence of coloration in each *A. arilaitensis* culture. Thus, this helped to conclude that hue phenotypes of the cultures are illustrations of the diversity among the *A. arilaitensis* strains.

Average and standard deviation of colorimetric coordinates of each *A. arilaitensis* cultures are shown in Table 2. Color measurements of these 14 *A. arilaitensis* cultures showed L^* values ranging from 31.47 to 50.51. They could be divided into 6 significantly different groups ($P < .05$) with *A. arilaitensis* AA028 presenting the lowest L^*, 31.47, while *A. arilaitensis* AA025 and WS2230 are gathered in a group presenting the highest L^*, 50.36 and 50.51, respectively.

The saturation, C^*, responses of 14 *A. arilaitensis* biofilms widely ranged from 2.08 to 14.80, whereby 9 groups of strains were statistically separated with distinct saturation ($P < .05$). Among the strains showing pale colored colonies, *A. arilaitensis* AA014 had the lowest C^* value. Besides these pale colored strains, 8 others displayed a significantly brighter yellow color (Fig. 2) with *A. arilaitensis* AA015 exhibited the brightest one. According to the analysis of pigment productions by the 14 *A. arilaitensis* strains (data not shown), 8 strains whose cultures presented high saturation values e.g. *A. arilaitensis* AA012, AA015, AA020, AA021, AA028, AA030, AA035 and CNRZ923, produced significantly higher quantities of pigments than the strains for which the C^* values of cultures were low. As pigments were encapsulated beneath cell-wall, this proof led us to conclude that C^* was responsible for interpretation in term of quantity of pigments produced by the *A. arilaitensis* strains.

As illustrated by colorimetric coordinates, several strains did have a significantly pale or cream color. This color was likely to be caused by cell material only. The results of pigment analysis using HPLC-DAD (data not shown) found that the extracts of cell biomass from all strains whose colonies were cream or pale colored, did not present the chromatographic profiles of carotenoids. For this reason, there is a possibility

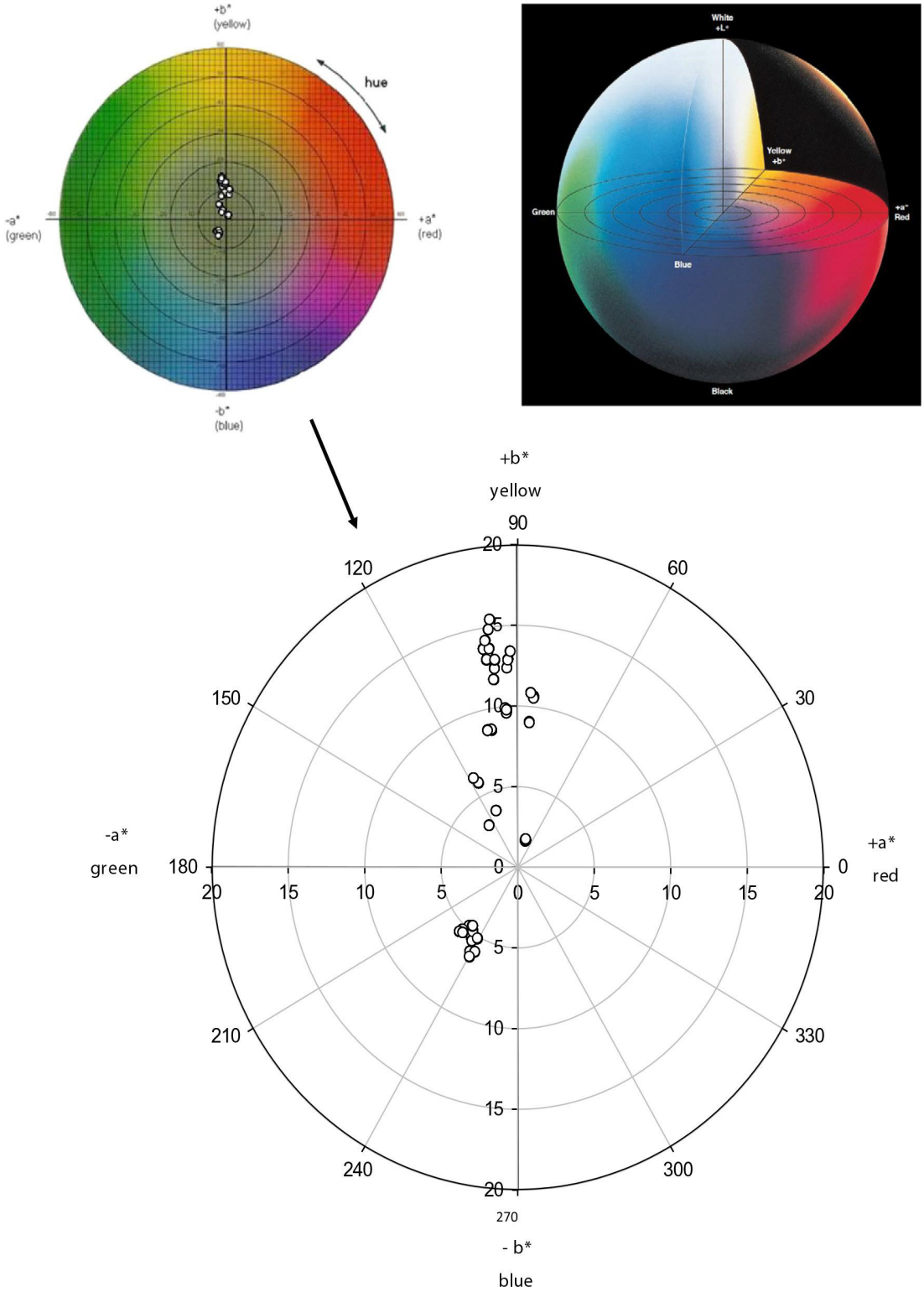

Fig. 2. Positions of *Arthrobacter arilaitensis* 14 strains in the CIE $L^*a^*b^*$ colorimetric system (strains cultivated in natural daylight, three replicates for each).

that these poorly or non-colored strains e.g. *A. arilaitensis* AA009, AA014, AA017, AA025, AA036 and WS2230, might be responsible only for producing a flavor but not for the coloration in cheeses due to the fact they do not produce any carotenoids. Deetae, Bonnarme, Spinnler, and Helinck (2007) described *A. arilaitensis* Mu107 as a strain belonging to a variety of ripened-cheese microbial communities and involved in the flavor formation through the production of volatile aroma compounds such as aldehydes, ketones and sulphur compounds. Our hypothesis from data obtained on milk ingredient-based agar on Petri dish should be further confirmed on cheese model with microbial interactions, and also directly on cheeses.

3.2. The effect of light on coloration of A. arilaitensis cultures

After 14 days of simultaneous incubation under either daylight or dark conditions, all replicates of *A. arilaitensis* strain were measured by

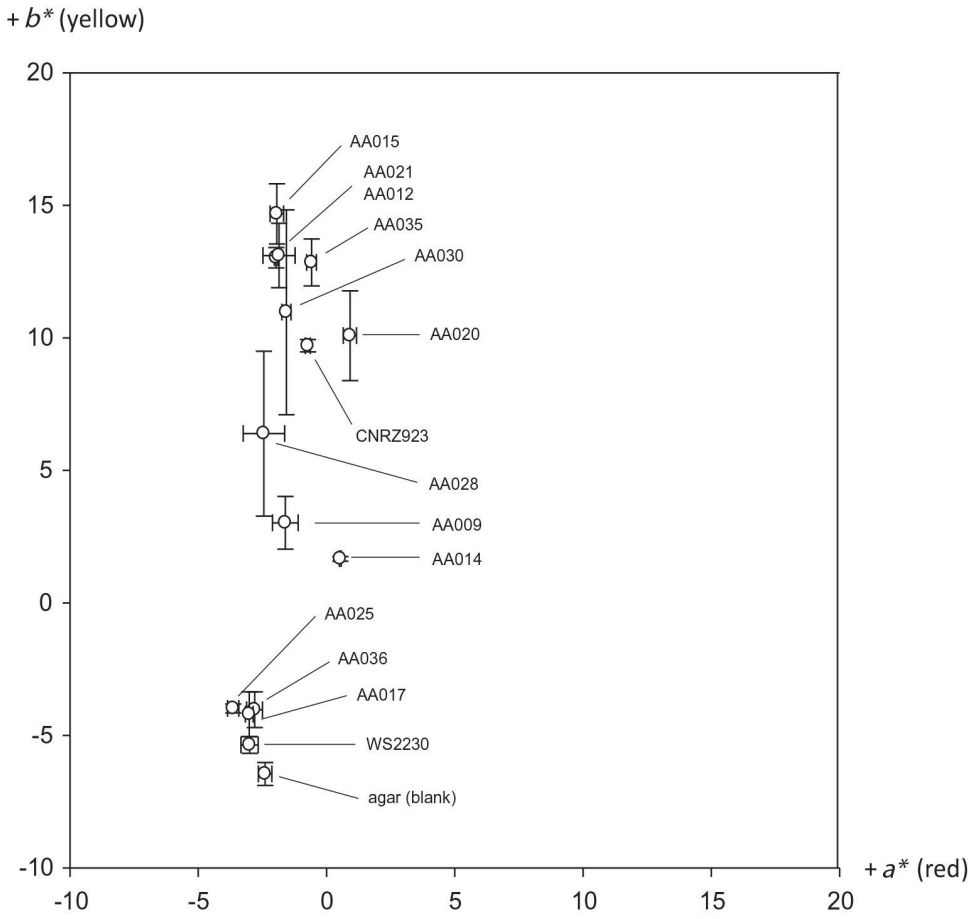

Fig. 3. Detailed positions of the 14 *Arthrobacter arilaitensis* strains, cultivated in daylight, when projected in the CIE $L^*a^*b^*$ colorimetric scale (bars indicate standard deviations).

a spectrocolorimeter as described in Section 2. Table 3 shows averages of each chromatic coordinates of 14 *A. arilaitensis* strains in darkness. When cultured in darkness, the a^* values ranged from −3.71 to 1.12 (versus −3.64 to 0.92 under daylight), and the b^* values varied from −3.66 to 3.88 (versus −5.36 to 14.64 under daylight). These could statistically significantly be divided into 9 and 7 groups, respectively ($P < .05$). Due to the scattered coordinates on (a^*, b^*) plane, an equation from correlation analysis could not be used to explain a relation between a^* and b^* values, as well as for the results under daylight condition.

When comparing data recorded either under dark or daylight conditions, two major qualitative differences could be remarked. First, the sets of experimental (a^*, b^*) pairs data obtained from *A. arilaitensis* cultivated in darkness had a more narrow range than the same data recorded under light condition (Figs. 3 & 4). The plots were located near the center of the chromatic plane. It meant that the C^* values of individual strains under the same condition were less discriminating when cultivated in darkness than in daylight; however, they could be separated into 9 groups by statistically significant differences ($P < .05$).

L^* values of *A. arilaitensis* ranged from 32.38 to 47.05 when cultivated under dark condition (against 31.47–50.11 under daylight). They could be statistically significantly divided into 9 groups ($P < .05$). *A. arilaitensis* AA025 and *A. arilaitensis* AA028 were strains which had the highest and the lowest values of lightness, respectively. Comparison between the two conditions demonstrated L^* values of cultures grown in daylight slightly wider ranged than those cultivated in darkness.

Table 2
Color coordinate values of the 14 *Arthrobacter arilaitensis* strains cultivated in daylight.

Strain	L^*	a^*	b^*	$h°$	C^*
A. arilaitensis AA009	40.15 ± 0.47^{d}	-1.61 ± 0.25^{d}	3.02 ± 0.50^{e}	118.52 ± 7.57^{d}	3.36 ± 0.30^{h}
A. arilaitensis AA012	44.74 ± 0.55^{b}	$-1.85 \pm 0.31^{d,e}$	13.11 ± 0.61^{b}	98.00 ± 1.06^{f}	13.24 ± 0.64^{b}
A. arilaitensis AA014	46.27 ± 0.11^{b}	0.54 ± 0.02^{b}	1.66 ± 0.05^{f}	72.39 ± 0.33^{h}	2.08 ± 0.14^{i}
A. arilaitensis AA015	42.87 ± 0.26^{c}	-1.94 ± 0.13^{e}	14.67 ± 0.57^{a}	97.55 ± 0.78^{f}	14.80 ± 0.55^{a}
A. arilaitensis AA017	40.09 ± 0.77^{d}	-2.80 ± 0.16^{g}	-4.03 ± 0.34^{g}	235.09 ± 3.63^{b}	4.91 ± 0.20^{g}
A. arilaitensis A020	37.09 ± 1.20^{e}	0.92 ± 0.13^{a}	10.08 ± 0.85^{c}	84.80 ± 0.52^{h}	$10.12 \pm 0.85^{c,d}$
A. arilaitensis AA021	46.08 ± 0.22^{b}	-1.96 ± 0.03^{e}	13.03 ± 0.19^{b}	98.57 ± 0.25^{f}	13.17 ± 0.19^{b}
A. arilaitensis AA025	50.36 ± 0.12^{a}	-3.64 ± 0.11^{h}	-3.98 ± 0.08^{g}	227.62 ± 0.95^{c}	$5.39 \pm 0.11^{f,g}$
A. arilaitensis AA028	31.47 ± 2.19^{f}	-2.44 ± 0.40^{f}	6.38 ± 1.56^{d}	112.09 ± 6.92^{e}	6.89 ± 1.35^{e}
A. arilaitensis AA030	39.13 ± 1.42^{d}	-1.56 ± 0.09^{d}	10.97 ± 1.93^{c}	98.42 ± 2.09^{f}	11.08 ± 1.88^{c}
A. arilaitensis AA035	$44.37 \pm 0.35^{b,c}$	-0.58 ± 0.10^{c}	12.84 ± 0.44^{b}	92.61 ± 0.51^{g}	12.86 ± 0.44^{b}
A. arilaitensis AA036	37.43 ± 0.30^{e}	-3.01 ± 0.08^{g}	$-4.20 \pm 0.42^{g,h}$	233.99 ± 3.59^{b}	$5.19 \pm 0.29^{f,g}$
A. arilaitensis CNRZ923	45.49 ± 0.41^{b}	-0.73 ± 0.05^{c}	9.7 ± 0.11^{c}	$94.28 \pm 1.58^{f,g}$	9.73 ± 0.12^{d}
A. arilaitensis WS2230	50.51 ± 0.27^{a}	-3.00 ± 0.17^{g}	-5.36 ± 0.16^{h}	240.83 ± 1.17^{a}	$6.15 \pm 0.19^{e,f}$

Values in the same column with a common superscript letter do not significantly differ ($P > .05$).

Table 3
Color coordinate values of the 14 *Arthrobacter arilaitensis* strains cultivated in darkness.

Strain	L^*	a^*	b^*	$h°$	C^*
A. arilaitensis AA009	40.51 ± 0.13^{f}	-1.71 ± 0.26^{e}	$2.73 \pm 0.32^{a,b}$	$122.23 \pm 6.82^{c,d}$	3.24 ± 0.14^{d}
A. arilaitensis AA012	44.80 ± 0.07^{c}	-0.36 ± 0.15^{d}	3.05 ± 0.07^{a}	96.77 ± 2.96^{e}	3.08 ± 0.05^{d}
A. arilaitensis AA014	42.27 ± 0.33^{e}	0.65 ± 0.07^{b}	1.98 ± 0.13^{d}	$72.63 \pm 0.31^{f,g}$	1.75 ± 0.04^{h}
A. arilaitensis AA015	42.17 ± 0.68^{e}	-1.28 ± 0.12^{f}	$2.42 \pm 0.18^{b,c,d}$	118.07 ± 3.90^{d}	$2.74 \pm 0.11^{e,f}$
A. arilaitensis AA017	39.46 ± 0.15^{g}	-3.14 ± 0.07^{h}	-1.95 ± 0.03^{f}	211.75 ± 1.05^{b}	3.69 ± 0.04^{c}
A. arilaitensis AA020	$39.86 \pm 0.18^{f,g}$	0.64 ± 0.04^{b}	0.71 ± 0.08^{e}	47.67 ± 2.44^{h}	0.96 ± 0.08^{i}
A. arilaitensis AA021	45.02 ± 0.41^{c}	$0.41 \pm 0.08^{b,c}$	$2.70 \pm 0.12^{a,b,c}$	81.43 ± 1.29^{f}	$2.74 \pm 0.13^{e,f}$
A. arilaitensis AA025	49.78 ± 0.47^{a}	-3.71 ± 0.14^{i}	-3.51 ± 0.50^{g}	$223.21 \pm 3.19^{a,b}$	5.12 ± 0.44^{a}
A. arilaitensis AA028	32.38 ± 0.03^{i}	-0.83 ± 0.41^{e}	$0.80 \pm 0.33^{e,f}$	134.45 ± 23.66^{c}	1.25 ± 0.12^{i}
A. arilaitensis AA030	$40.26 \pm 1.11^{f,g}$	-0.82 ± 0.26^{e}	$2.22 \pm 0.05^{c,d}$	110.13 ± 5.45^{d}	2.38 ± 0.13^{g}
A. arilaitensis AA035	43.59 ± 0.14^{d}	1.27 ± 0.09^{a}	$2.77 \pm 0.89^{a,b}$	65.30 ± 2.22^{g}	$3.05 \pm 0.05^{d,e}$
A. arilaitensis AA036	36.66 ± 0.76^{h}	-3.23 ± 0.19^{h}	-3.19 ± 0.58^{g}	224.27 ± 7.02^{a}	4.56 ± 0.26^{b}
A. arilaitensis CNRZ923	45.61 ± 0.41^{c}	0.28 ± 0.08^{c}	$2.57 \pm 0.15^{a,b,c}$	83.83 ± 1.58^{f}	$2.59 \pm 0.15^{f,g}$
A. arilaitensis WS2230	47.19 ± 1.31^{b}	-3.61 ± 0.18^{i}	-3.66 ± 0.57^{g}	225.12 ± 5.15^{a}	5.16 ± 0.37^{a}

Values in the same column with a common superscript letter do not significantly differ ($P > .05$).

In contrast to the lightness, $h°$ values of cultures grown under dark condition had wider variations than the strains cultivated under light. *A. arilaitensis* AA020 displayed the lowest hue angle, 47.67°, while *A. arilaitensis* WS2230 showed the highest $h°$ value, 225.12°.

The second difference concerns the individual ranks of the 14 strains between cultures grown in daylight and in darkness. Comparison of Fig. 3 with Fig. 4 shows that the ranking of the strains changed according to the light conditions. The color of the majority of the strains became paler when cultivated in darkness whereas one strain was less colored when cultivated under light. The color of cultures between the 2 conditions was strongly different for the strains AA012, AA015 (Fig. 5), AA020, AA021, AA028, AA030, AA035 and CNRZ923 with C^* values higher when cultivated in daylight. Although saturation of the 14 *A. arilaitensis* strains was statistically significantly different among the same cultivated condition (daylight or darkness), the C^* value difference for individual strains cultivated in darkness was excessively lower when comparing with daylight condition.

When considering L^* and $h°$ values, light influenced on the change of some strains. Light had statistically significant effect on the lightness of the cultures of 5 strains, while 10 strains were affected by light on their hues.

Three different types of color response in term of (a^*, b^*) pairs were statistically separated. Group 1 was composed of strains AA012, AA015, AA017, AA020, AA021, AA028, AA030, AA035, AA036, CNRZ923 and WS2230. These displayed brighter pigmentation under daylight condition than in the darkness and were stated to be positively sensitive to

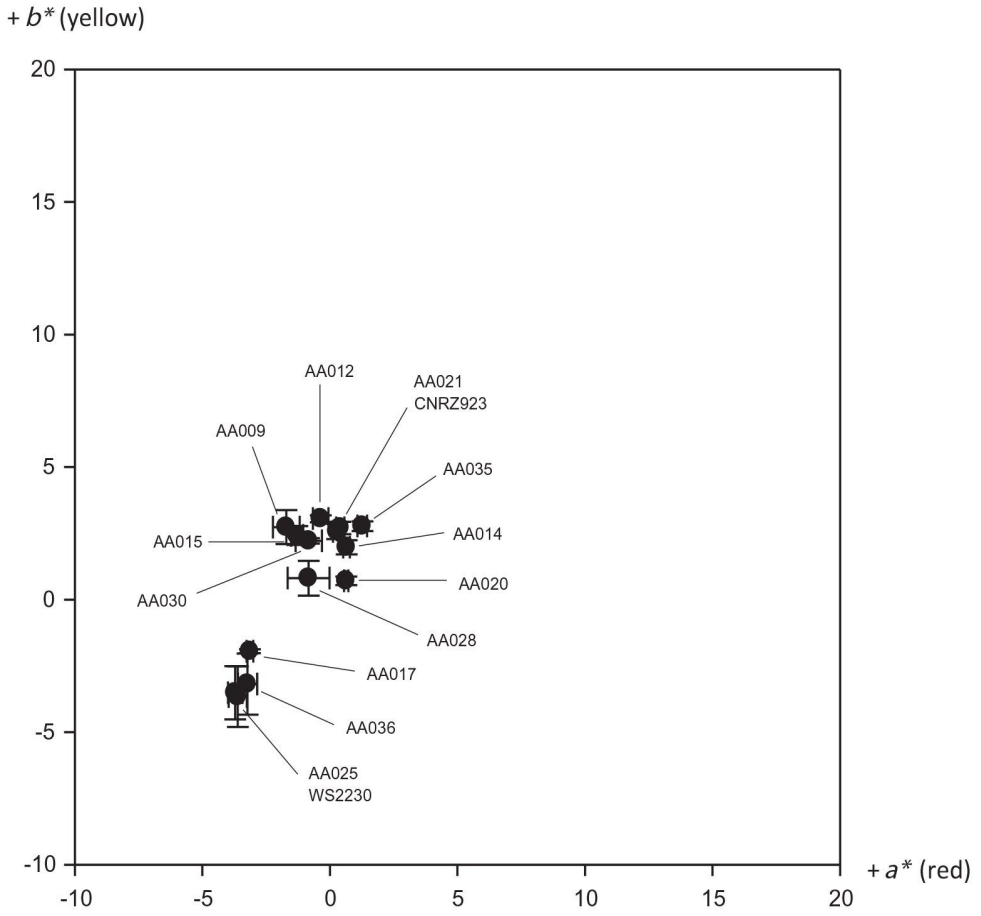

Fig. 4. Detailed positions of the 14 *Arthrobacter arilaitensis* strains, cultivated in darkness, when projected in the CIE $L^*a^*b^*$ colorimetric scale (bars indicate standard deviations).

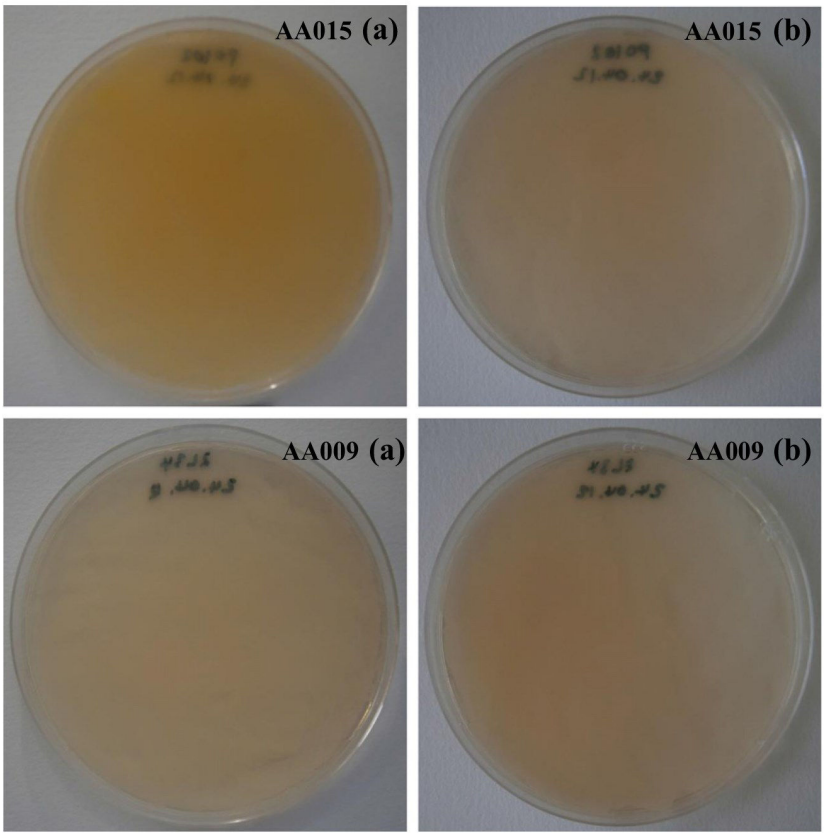

Fig. 5. Coloration of *Arthrobacter arilaitensis* strains AA015 and AA009 cultivated on milk-ingredient based medium (Petri dish) under two conditions: daylight (a) and darkness (b).

the light. Group 2 combined *A. arilaitensis* AA009 and AA025. They had practically the same colorimetric coordinates in both conditions and were regarded as non-sensitive to the light factor. Group 3 included only one strain of *A. arilaitensis*, AA014, which displayed paler pigmentation under light than when cultivated in darkness. It was considered to be negatively sensitive to the light. Fig. 6 shows the examples of typical coloration behavior of *A. arilaitensis* affected by light exposure; *A. arilaitensis* AA015 (positively sensitive), *A. arilaitensis* AA025 (not sensitive) and *A. arilaitensis* AA014 (negatively sensitive). Therefore, light could be considered as a factor affecting the pigmentation of *A. arilaitensis*. This concerns not only the color but also the saturation, particularly for the strains whose colonies were intense yellow in color such as *A. arilaitensis* AA012, AA015, AA020, AA021, AA028, AA030, AA035 and CNRZ923. The study on bacteriology of Limburger cheese reported that light did not affect the pigmentation of the white, cream, gray-white and red *Arthrobacter* strains isolated from this kind of cheese (El-Erain, 1969). Under both dark and light conditions, most of the 140 such strains were found to present the same color. Only the pigmentation of greenish-yellow *Arthrobacter* was affected by light: a gray-white or light cream yellow color was observed when cultivated in the dark, while in the light the colonies were greenish-yellow (El-Erain, 1969).

The light-positive response in this experiment could be explained by some light-induced carotenogenesis (Takano, Asker, Beppu, & Ueda, 2005) as the expected pigments produced by *A. arilaitensis* isolated from cheeses are carotenoids. Recently, Galaup et al. (2007) reported that *A. arilaitensis*, one of the major bacterial species found at the surface of smear-ripened cheeses, produced a yellow pigment which was tentatively identified as a carotenoid mixture. Furthermore, the carotenoids may belong to the C_{50}-subfamily according to the study of Monnet

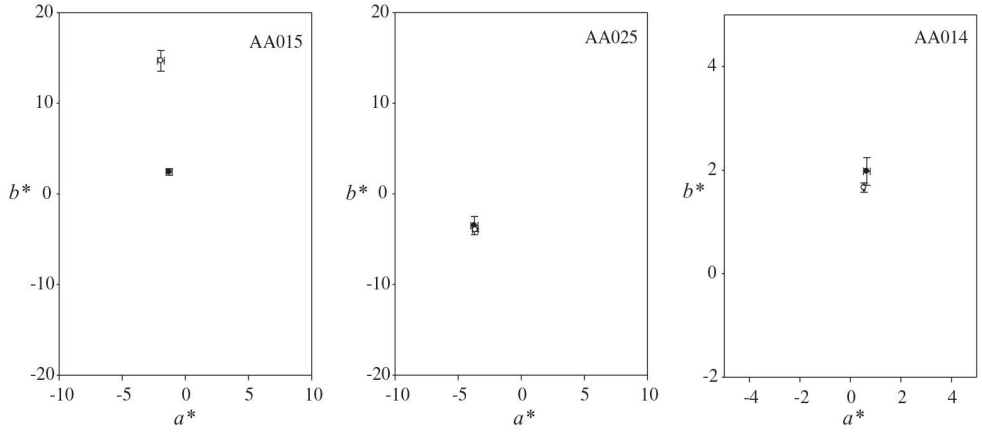

Fig. 6. Effect of light exposure on coloration behavior of *Arthrobacter arilaitensis* strains: AA015: positively sensitive, AA025: not sensitive; AA014: negatively sensitive. ● = darkness, ○ = daylight (bars indicate standard deviation).

et al. (2010). Although the role of carotenoids produced by *A. arilaitensis* has not been revealed, one function of carotenoids in microorganisms is to serve as membrane integrated antioxidants, protecting cells from oxidation stress (Krinsky, 1989; Liu, Gai, Tao, Tang, & Xu, 2012). In the genus *Arthrobacter*, however, several previous studies have reported a relation between carotenoids produced by some members of this genera and their living conditions. According to Fong, Burgess, and Barrow (2001), changes of carotenoids production in response to growth temperature and salt concentration provided insights into the adaptation to the cold environment of the psychrotrophic *Arthrobacter agilis*, a bacteria isolated from Antarctic sea ice. Pigments may therefore be associated to stabilization of cell membrane at low temperature. Moreover, the survival of some microorganisms at low temperature may be enhanced by the ability of carotenoid to rigidify membranes (Shivaji & Ray, 1995). Dieser, Greenwood, and Foreman (2010) also indicated that carotenoid pigmentation increases the resistance of the heterotrophic bacteria, *A. agilis* MB8-13, to environmental stressors, being cryo and solar radiation protectants. It is generally recognized that carotenoid pigments provide antioxidant protection through quenching, by being oxidized or through co-oxidization by light-excited photosentizers with strong oxidant power.

The occurrence of one *A. arilaitensis* strains, AA014, which was brighter in the dark than in daylight did not follow this theory. As its colonies were pale colored and the result of pigment analysis using HPLC (data not shown) did not exhibit any chromatographic profile of carotenoids (data not shown), it was clear that *A. arilaitensis* AA014 did not produce yellow carotenoids. Thus, one possible explanation would be that the color was likely to be caused by cell material only, and that light might affect its growth and biomass production.

4. Conclusions

An obvious diversity of microbial behaviors was discovered through this experiment. Alignments of hue value from each experimental (a^*b) pair ranged from 72.39 to 240.83° and could be statistically divided into 9 groups. The major group was composed of 5 strains showing close $h°$ responses ranging from 92.61 to 98.57 while the remaining groups consisted in a single or two strains only. However, 8 strains of *A. arilaitensis*, among 14, presented yellow to greenish-yellow pigments in their cultures, while the others showed cream or pale color. Light exposure affected pigmentation of these strains not only hue but also saturation and lightness. Three groups depending on coloration behavior consecutive to light impact were illustrated among the 14 *A. arilaitensis* strains studied e.g. positively sensitive, negatively sensitive and not sensitive to light.

Author contributions

N. Sutthiwong and L. Dufossé designed the study. N. Sutthiwong performed the experiments. N. Sutthiwong, Y. Caro, C. Milhau, A. Valla, M. Fouillaud and L. Dufossé interpreted the results. N. Sutthiwong drafted the manuscript. Y. Caro, A. Valla, M. Fouillaud and L. Dufossé participated in revising the article critically.

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CHAPITRE 5

Influence de l'environnement dans la production de pigments par *Arthrobacter arilaitensis*

La couleur du fromage à pâte molle et croûte lavée est due au développement d'une microflore de surface, généralement composée de levures et de bactéries. Le phénomène de coloration des fromages dans cette famille est d'autant plus complexe que quelques interactions entre les micro-organismes dans le biofilm peuvent se produire; par ailleurs, les facteurs de l'environnement, des facteurs physiques et chimiques, affectent également la coloration de fromages à croûte lavée.

Au cours des dernières années, *A. arilaitensis* a été identifié comme une souche bactérienne importante responsable de la coloration des fromages à croûte lavée; toutefois, à notre connaissance, seule une étude s'est consacrée à la capacité de coloration de cette espèce sur un seul facteur, la variété de milieux déacidifiés. D'autre part, plusieurs études antérieures ont été conduites sur *Brevibacterium linens* sur la coloration du fromage selon l'effet de divers facteurs tels que le pH, la concentration en sel, l'humidité, la lumière, la température et les levures utilisées dans la désacidification ainsi que leur interaction avec d'autres micro-organismes isolés à partir de fromages affinés.

Ce chapitre comprend des résultats expérimentaux des articles 5 et 7 du chapitre 4, présentant l'influence de la lumière sur la production de pigment et la caractérisation d'*Arthrobacter arilaitensis*, ainsi que le développement de la couleur de cette bactérie sous les effets conjugués du pH, du NaCl et des levures utilisées dans la désacidification.

Influence of light on biomass and pigment production of carotenoid-producing strains (Dans l'article 5)

After the simultaneous incubation in milk ingredient-based medium under either light or dark conditions, cultures of 8 carotenoids-producing strains were collected and their biomass and pigment productions further determined. When cultured these strains in darkness, the dry biomass values ranged from 2.32 to 3.66 versus 2.58 to 3.84 under light condition; however, within individual strains, there was not a statistically significant difference between the 2 conditions used for cultivation ($P < .05$).

In contrast to the biomass production, carotenoids produced by carotenoids-producing strains grown under light condition had higher pigment concentrations than the strains cultivated under dark condition. When cultivating in the light, carotenoid production varied between 0.40 to 0.76 mg L^{-1} with *A. arilaitensis* AA012 displaying the lowest carotenoids production, 0.40 mg L^{-1}, while *A. arilaitensis* AA028 presented the highest carotenoids content, 0.76 mg L^{-1}. The range of carotenoid production in the darkness was between 0.17 to 0.25 mg L^{-1}. After statistical analysis, carotenoids quantities produced by the same strains but grown in the different conditions were significantly different ($P < .05$).

The results led to conclude that light influenced the ability to produce pigments in all yellow-colored *A. arilaitensis* strains studied, but had no impact on their biomass production.

The effect of light on coloration of *Arthrobacter arilaitensis* cultures (dans article 7)

Spectrocolorimetry was applied to characterize the pigmentation of *A. arilaitensis*. When comparing data recorded either under dark or daylight conditions, two major qualitative differences could be remarked. First, the sets of experimental *(a*, b*)* pairs data obtained from *A. arilaitensis* cultivated in darkness had more narrow range than the same data recorded under light condition. The plots were located near the center of the chromatic plane. It meant that the *C** values of individual strains under the same condition were less discriminating when cultivated in darkness than in daylight.
Comparison between the 2 conditions presented that *L** values of cultures grown in daylight slightly wider ranged than the cultivation in darkness, while h^o values of cultures grown under dark condition had wider varying than the strains cultivated under light.

The second difference implicated the individual ranks of the 14 strains between cultures grown in daylight and in darkness. The ranking of the strains when projected onto a (*a**, *b**) plane changed according to the light conditions. The color of the majority of the strains changed to be paler when cultivated in darkness whereas certain strains were less colored when cultivated under light. Moreover, the color of cultures between the 2 conditions were exceedingly different, which *C** values were higher when cultivated in daylight. Although saturation of the 14 *A. arilaitensis* strains was statistically significantly different among the same cultivated condition, (daylight or darkness), the *C** value difference for individual strains cultivated in darkness was excessively lower when comparing with daylight condition.

Three different types of color response in term of (*a**, b*) pairs were statistically significantly separated. Group 1 displayed a brighter pigmentation under daylight condition than in the darkness, and was stated to be positively sensitive to the light. Group 2 had practically the same colorimetric coordinates in both conditions, and was regarded as non-sensitive to the light factor. Group 3 showed paler pigmentation under light than cultivated in darkness. It was considered to be negatively sensitive to the light. Therefore, light could be considered as a factor affecting the pigmentation of *A. arilaitensis*. The light-positive response in this experiment could be explained by some light-induced carotenogenesis. Although a role of carotenoids produced by *Arthrobacter arilaitensis*, has not particularly been revealed, one function of carotenoids in microorganisms is to serve as membrane integrated antioxidants, protecting cells from oxidation stress.

Article 8

☒ Color development of cheese-ripening bacteria *Arthrobacter arilaitensis* under the influence of pH, NaCl and deacidifying yeasts, *Debaryomyces hansenii* and *Kluyveromyces marxianus*. **Sutthiwong N**., Dufossé L.. (Soumission prévue à une revue internationale).

La couleur de la surface des fromages à croûte lavée est l'un des principaux attributs qualitatifs de ces produits. Elle est le résultat de la synthèse de pigments par une microflore complexe, composée de diverses espèces de bactéries et de levures. Pendant le processus de production du lait cru vers les fromages, des communautés microbiennes complexes s'expriment.

L'affinage de surface commence avec la croissance des levures, en fonction de la variété de fromages, principalement *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica* et *Geotrichum candidum*, qui colonisent la surface du fromage et assimilent l'acide lactique au cours de la période initiale de maturation. Cette assimilation induit une augmentation du pH de la surface, facilitant la croissance de bactéries sensibles aux acides. Par conséquent, le développement des bactéries sur la surface du fromage semble être dépendant du métabolisme de l'acide lactique par des levures. Hormis les levures utilisées dans la désacidification du fromage, d'autres facteurs tels que la lumière, le pH, la température et le NaCl peuvent également influencer la pigmentation des bactéries de fromages affinés.

Arthrobacter arilaitensis est une espèce bactérienne émergente responsable de la coloration de surface des fromages à pâte molle et croûte lavée; par consequent, il n'y a pas encore beaucoup d'informations sur cette espèce en association avec la coloration des fromages. La présente étude a été réalisée afin d'étudier les influences du pH, du NaCl et des levures de désacidification en combinaison, sur la pigmentation de cette bactérie.

Color development of cheese-ripening bacteria *Arthrobacter arilaitensis* under the influences of pH, NaCl and deacidifying yeasts, *Debaryomyces hansenii* and *Kluyveromyces marxianus*

Short title:

Color of *A. arilaitensis* influences by pH, NaCl and deacidifying yeasts

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Abstract

Arthrobacter arilaitensis is an emerging bacterial species responsible for the coloration of surface-ripened cheeses; therefore, there is still not much information about this species in association with cheese coloration. The present study was performed in order to investigate the influences of pH, NaCl and deacidifying yeasts on pigmentation of *Arthrobacter arilaitensis* Po102 using spectrocolorimetry. Three types of cheese-based solid media were prepared depending on deacidification method studied: (i) chemical deacidification by adding NaOH (CM_{NaoH}); and (ii) biological deacidification by inoculating yeast strain either *Debaryomyces hansenii* 304 (CM_{Dh304}) or *Kluyveromyces marxianus* 44 (CM_{Km44}), respecting initial pH values required i.e. 5.8, 7.0, and 7.5. After pasteurizing and adding NaCl (0%, 2%, 4% and 8% (w/v)), *A. arilaitensis* was inoculated on the deacidified media and incubated at 12 ^{o}C under light condition for 28 days.

A diversity of colors provided by *A. arilaitensis* appeared among three types of deacidified media, as well as among the same type, broadening from pale to intense yellow. Initial pH 5.8 and 8% (w/v) NaCl showed the efficiency inhibited effect on pigmentation and growth of *A. arilaitensis* in all types of deacidified media. In combination with pH and NaCl, deacidifying yeasts were obviously related to the pigment production of *A. arilaitensis*. At pH 7.0 and 7.5, the color development by *A. arilaitensis* Po102 on CM_{Dh304} was higher than on CM_{Km44}. The highest average value of color saturation were observed on CM_{Dh304} at pH 7.0, showing intense yellow; although the highest color saturation of pigment produced by *A. arilaitensis* presented on CM_{Dh304} at pH 5.8 with 0% (w/v) NaCl.

Keywords: Arthrobacter arilaitensis; Pigmentation; Surface-ripened cheese; Spectrocolorimetry

1. Introduction

Surface color of smear-ripened cheeses is one of the major attributes determining a consumer's purchasing decision since it is a potential representative of many qualities e.g. maturity, naturalness and flavor. A large number of smear-ripened cheeses, for example, Munter, Livarot and Reblochon from France, Limberger and Tilsit from Germany, and Gubbeen and Taleggio from Italy, are recognized by a consumer through the presence of color on rind, which depend on the appropriate color of each cheese.

The surface of smear-ripened cheeses belongs to a result of pigment synthesis by a complex surface microflora, consisting of diverse species of bacteria and yeasts. Study on the surface microflora of smear-ripened cheeses is always in the interest of microbiologists, as microbial populations are not yet well-known; therefore, several novel microbial strains have been discovered as important as *Brevibacterium linens*, which was previously considered to be a sole major microorganism responsible for the color development, for the ripening of the cheese rind (Brennan et al., 2002; Fontana, Cappa, Rebecchi, Cocconcelli, 2010; Larpin-Laborde et al., 2011; Mounier et al., 2008) . One among these microorganisms is *Arthrobacter* sp. due to it has been found at different stages of ripening. According to Bockelmann (2002), the color development of typical light-brown cheese was attributed to the interactions between yellow-pigmented *Arthrobacter* sp. and other microorganisms while the orange pigments *B. linens* were found lesser important, similar to the results of Eliskases-Lechner and Ginzinger (1995). More recently, *Arthrobacter arilaitensis* has been identified from smear-ripened cheeses, as one of important bacteria among a diversity of microorganisms found during ripening, particularly at the middle and late stages of ripening (Bockelmann, 2002; Feurer, Vallaeys, Corrieu, Irlinger, 2004; Irlinger, Bimet, Delettre, Lefèvre, Grimont, 2005; Larpin-Laborde et al., 2011).

During production process from raw milk to become cheeses, it provides complex microbial communities. Surface ripening begins with the growth of yeasts, depending on the variety of cheeses, mainly *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica* and *Geotrichum candidum*, which colonize the cheese surface and assimilate lactic acid during the initial period of ripening (Corsetti, Rossi, Gobbetti, 2001; Mounier et al., 2008) . This assimilation induces an increase of the surface pH, facilitating the growth of acid-sensitive bacteria. Consequently, the development of bacteria on cheese surface appears to be dependent on the metabolism of lactic acid by yeasts (Leclercq-Perlat, Oumer, Bergere, Spinnler, Corrieu, 2000; Valdès-Stauber, Scherer, Seiler, 1997). Deacidifying yeasts have been reported to affect both the growth and the pigmentation of smear-ripening bacteria. According to Leclercq-Perlat, Courrieu and Spinnler (2004), the color intensity of pigment produced by *B. linens* was higher when using *D. hansenii* for deacidification than *K. marxianus*. In addition, significant differences of the color intensity provided by ripening bacteria in combination with different

strains of *D. hansenii* have been demonstrated (Masoud, Jakobsen, 2003). Besides yeasts used in cheese deacidification, other factors such as light, pH, temperature and NaCl can also influence on pigmentation of cheese-ripening bacteria. Masoud and Jakobsen (2003) reported the significant effects of pH and NaCl on the intensity of pigment produced by *B. linens* and *Corynebacterium flavescens*. In the same study, the strongest color intensity presented at pH 7.0, contrasting with pH 6.0 and pH 5.6; meanwhile the addition of 4% (w/v) NaCl resulted in the highest intensity of the orange-reddish color when compared with 0 and 8 %.

To date, an interested aspect concerning with the color development by the surface microflora stimulates the augmentation of research projects on cheese coloration under the influences of various factors e.g. interactions between the microorganisms in the smear and factors affecting the environment for microbial growth, including physical and chemical factors. Nevertheless, the coloration by *A. arilaitensis* seems not to often be investigated, especially its pigmentation affected by various factors, in contrast to the orange *B. linens* strains. The present study has been performed in order to investigate the effects of three factors in combination i.e. initial pH, additional NaCl and deacidifying yeasts on the pigmentation by *A. arilaitensis* using quantitative spectrocolorimetry.

2. Materials and methods

2.1 Strains and cultures

2.1.1 Yeast and bacterial strains

Yeast strains used in this study i.e. *Debaryomyces hansenii* 304 and *Kluyveromyces marxianus* 44 were kindly provided by the Institut National de la Recherche Agronomique (INRA), France, while a bacterial strain, *Arthrobacter arilaitensis* Po102, was isolated from cheeses in our laboratory. Usually stored at -80 °C, they were maintained during this study on medium depending on their nutrient requirements, stored at 4 °C and subcultured monthly.

2.1.2 Cultures

Potato Dextrose Broth (BD Difco) was used as a subculture medium for yeasts while the milk ingredient-based medium was prepared and used for bacteria. The milk ingredient-based medium containing 5 g Casamino acids (BD Difco), 1 g yeast extract (BD Bacto), 5 g NaCl (Fisher Scientific), 20 g D-Glucose (Fisher Scientific), 1 g KH_2PO_4 (Fisher Scientific) and 15 g agar granulated (BD Difco) per liter of dionized water was prepared. Before sterilizing at 121 °C for 15 min, the pH of medium was adjusted to 7.0 ± 0.2.

To prepare a microbial suspension, each of *D. hansenii* 304 and *K. marxianus* 44 was inoculated into 250 ml Erlenmeyer flask containing 50 ml of PDB whilst the milk ingredient-based medium was used instead of PDB for *A. arilaitensis* Po102. These flasks were incubated at 25 °C with agitating at 150 rpm for 2 days. After cultivation period, cells were harvested by centrifuging at 6000 x *g* for 10 min, and re-suspended in peptone saline diluent [1 g casein peptone (Sigma) and 8.5 g NaCl (Fisher Scientific), adjusted pH to 7.0 ± 0.2 at 25 °C)]. A suspension containing 10^7 cells mL^{-1} was prepared to use in the next step.

2.2 Deacidified cheese-based media and factors controlled

The procedure for preparing the experimental media employed in this study was adapted according to Leclercq-Perlat, Corrieu and Spinnler (2004), Rea et al. (2005) and Masoud and Jakobsen (2003). The cheese-based media were prepared in two steps: (*i*) pH adjustment by chemical or biological deacidification, depending on the observation of coloration influenced by yeasts; and (*ii*) inoculation of bacterial strains on the media surface.

The cheese curd used throughout the experiment was kindly supplied by Fromagerie de Bourbon, Reunion Island, France. After obtaining, the cheese curd was frozen at -80 °C for preventing enzymatic reaction (Martínez-Cuesta, Palencia, Requena, Pelácz, 2001; Swearingen, O'Sullivan, Warthsen, 2001).

Before preparing cheese-based media, roughly 24 h, the frozen cheese curd was thawed at 4 °C. The defrost cheese curd was mixed with distilled water (60 % w/v); then the mixture was homogenized using a blender and placed in a water bath (100°C) for 30 min. After cooling down to 60 °C, the mixture was blended again until the texture was homogeneous. This cheese slurry was divided depending on the volume needed for each treatment and added into sterilized flasks for further steps, the pH adjustment.

2.2.1. pH adjustment

2.2.1.1 Chemical deacidification

A flask containing cheese slurry was adjusted pH to 5.8, 7.0 or 7.5, with 1M NaOH (Fisher Scientific), followed by the addition of 2 g agar and NaCl (0%, 2%, 4% or 8% (w/v)). The final mixture was pasteurized by heating at 80°C for 10 min. After cooling down to 45 – 50 °C, pour plate technique was applied to prepare the cheese-based solid medium contained in Petri-dishes, which were left to solidify for 2 h before inoculating with *A. arilaitensis*.

2.2.1.2 Biological deacidification

When the temperature of the cheese slurry decreased close to 30 °C, a flask of the slurry was inoculated by either *D. hansenii* or *K. marxianus*, through the suspension containing 10^7 cells mL^{-1}, followed by homogenizing awhile. Then, the flask was wrapped by aluminum foil, accepted a silicone cork which used for closing the flask, and incubated in the darkness at 25 °C with agitating 150 rpm until the pH values increased to 5.8, 7.0 or 7.5.

For stopping the activity of yeast to affect pH value, the cheese slurry was heated at 80 °C for 10 min to eliminate the yeast cells. An amount of agar including NaCl, depending on each treatment, was added to the mixture. Afterward, the procedures as mentions above in chemical deacidification, after adjusting pH by chemical reagent, were applied.

2.2.2 Inoculation of Arthrobacter arilaitensis and cultivation conditions

After solidifying, one ml of a 10^7 cell suspension of *A. arilaitensis* was spread over the surface of the deacidified media. All Petri-dishes were then incubated at 12 °C under light. Three independent replicates for each treatment were collected and measured using spectrocolorimetry for the values of color coordinates at d7, d14, d21 and d28.

2.4 Color measurements

The cheese-based solid medium inoculated with *A. arilaitensis* Po102 was measured using CM-3500d spectrocolorimeter (Minolta Co., Ltd., Japan) driven with SpectraMagic *NX* Pro. software (Minolta). The reference illuminant was D65 (standard daylight). According to the Compagnie Internationale de l'Eclairage (CIE, 1978) the data were reported in the *L*a*b** colorimetric system. A single-pieced disk of agar, large enough to cover the entire light spot, was cut in each Petri dish, from an area of homogeneous colony development. The sample was then hold culture-down and layered in the bottom of a 45 mm diameter CM-A128 glass Petri dish (Minolta) for incident light color measurements.

To characterize a color in the CIE *L*a*b** color system, 3 colorimetric coordinates are obtained from the spectrocolorimeter. *L** defines lightness (ranges from 0% to 100%, dark to light), *a** indicates the red/green value (from -60 to 60, green to red), and *b** denotes the blue/yellow value (from -60 to 60, blue to yellow). The attributes of color, *C** and h°, describe the chroma (vividness or dullness) and the hue angle or tone of the color, respectively. The value of chroma *C** is 0 at the center and increases according to the distance from the center. Hue angle h^o is defined as starting at the $+a^*$ axis and is expressed in degree: 0° would be $+a^*$ (red), 90° would be $+b^*$ (yellow), 180° would be $-a^*$ (green), and 270° would be $-b^*$ (blue). Hue values correspond to the angle of the a^*/b^* coordinated of the points.

$$\text{Chroma } C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$\text{Hue angle } h^o = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

2.5 Statistical analysis

The data were analyzed using SigmaPlot software (Systat Software, Inc., USA). To determine the color coordinate values of the biofilms produced by *A. arilaitensis* affected by initial pH, additional NaCl concentration and deacidifying yeasts, one-way analysis of variance (one-way ANOVA) was performed for comparing mean values among the whole data of each factor, while multivarirate analysis of variance (MANOVA) was applied when determining the combined effects of controlled factors on the color of *A. arilaitensis* biofilms. The differences of considered variable were estimated by Turkey and Holm-Sidak tests according to a α risk of 5% and 1% for one-way ANOVA and MANOVA, respectively.

3. Results

3.1 Diversity of coloration by Arthobacter arilaitensis on different deacidified media

After 28 days of simultaneous incubation at 12°C under light condition, the cheese-based solid media inoculated with *A. arilaitensis* Po102 were measured color attributed. Among three media prepared depending on the method used for deacidification, i.e., adjusted pH by NaOH (CM_{NaOH}), *D. hansenii* 304 (CM_{Dh304}) and *K. marxianus* 44 (CM_{Km44}), the coloration of strain Po102 on CM_{NaOH} showed alignments closer to the 90° hue value than those of CM_{Dh304} and CM_{Km44}; furthermore, a large number of the a^* values were negative, extending from -2.50 to 3.30, while the b^* value varied between 6.00 and 29.30 (data not shown). This led the most of inoculated CM_{NaOH} displayed a pale color, in contrast to CM_{Dh304} and CM_{Km44}. The a^* values of *A. arilaitensis* biofilms on CM_{Dh304} and CM_{Km44} were varying from -2.50 to 6.05 and -2.08 to 3.70, with b^* values ranging from 80.50 to 105.85 and 82.10 to 105.55, respectively (data not shown). These experimental (a^*, b^*) pairs were brought into divergent alignments with the hues enlarging from 80.50 to 105.85° and 82.10 to 105.55 for CM_{Dh304} and CM_{Km44}, respectively. The results indicated a diversity of colors among these deacidified media, particularly between CM_{NaOH} and CM_{Dh304} whose h° values were statistically significantly different ($P < .05$).

The color detail positions of biofilms produced by *A. arilaitensis* on $CM_{NaOH,}$ CM_{Dh304} and

CM_{Km44} coordinating with initial pH and concentration of NaCl are shown in Fig. 1. When considering the color projected onto a (a^*, b^*) plane among the same deacidification method, the cheese-based agar with pH 5.8 was significantly different from the other ones with pH 7.0 and 7.5 ($P<.05$), while there was not a significant difference between pH 7.0 and pH 7.5 ($P>.05$). However, the color among the whole set of the pH 7.0 were wider distributed on the (a^*, b^*) plane than those of pH 5.8 and 7.5 in all types of deacidified media. When cultured *A. arilaitensis* on each media, excluding in the absence of NaCl, a narrow range of color coordinate values was distinctly observed on three types of deacidified media at pH 5.8, corresponding to a similar position detected from the blank agar.

3.2 The combined effects of pH, NaCl and deacidification method on the pigmentation of Arthrobacter arilaitensis

3.2.1 Changes in color development of Arthrobacter arilaitensis during cultivation

The color characteristic in terms of hue angle (h^o) and chroma (saturation, C^*), under the influences of pH, NaCl concentration and deacidification method, are shown in Table 1 and 2, respectively. After testing the analysis of variance, there was a significant interaction between these variables on the hue and chroma of the biofilms produced by *A. arilaitensis* ($P<.001$). The hue angles extended from 80.53 to 105.74, with a large number of data showing responses close to h^o value obtained from the blank agar, which corresponded to a cream or pale color (poorly colored). Among the same type of media, h^o values decreased when cultivated on agars adjusted pH 7.0 and pH 7.5, leading to display more colored biofilms than those of pH 5.8, especially on CM_{Dh304} at pH 7.5 which presented an intense yellow at all levels of NaCl concentration. Although *A. arilaitensis* was likely to not able to form color when the amount of NaCl was hugely added into these media at pH 5.8, the biofilms on all types of media displayed a yellow color in the absence of NaCl, showing 87.04, 80.53 and 82.20^o hue values for CM_{NaOH}, CM_{Dh304} and CM_{Km44}, respectively, which significantly differed from the value of the blank agar ($P<.05$).

Similarly the hue, the factors including pH, concentration of NaCl and deacidifiation method affected the saturation, C^*, of *A. arilaitensis* biofilms. The C^* responses of these biofilms widely ranged from 6.56 to 38.54, whereby a large diversity of different groups was significantly separated ($P<.001$). Although CM_{Dh304} at pH 5.8 without adding NaCl had the highest C^* value, the other ones on this medium with higher NaCl content were very low, which were identical to the blank agar. Regardless of NaCl concentration, the highest average saturation of color appeared on CM_{Dh304} at pH 7.0 ($C^* = 29.48$), while the lowest average C^* value displayed on CM_{NaOH} at pH 5.8 ($C^* = 14.02$).

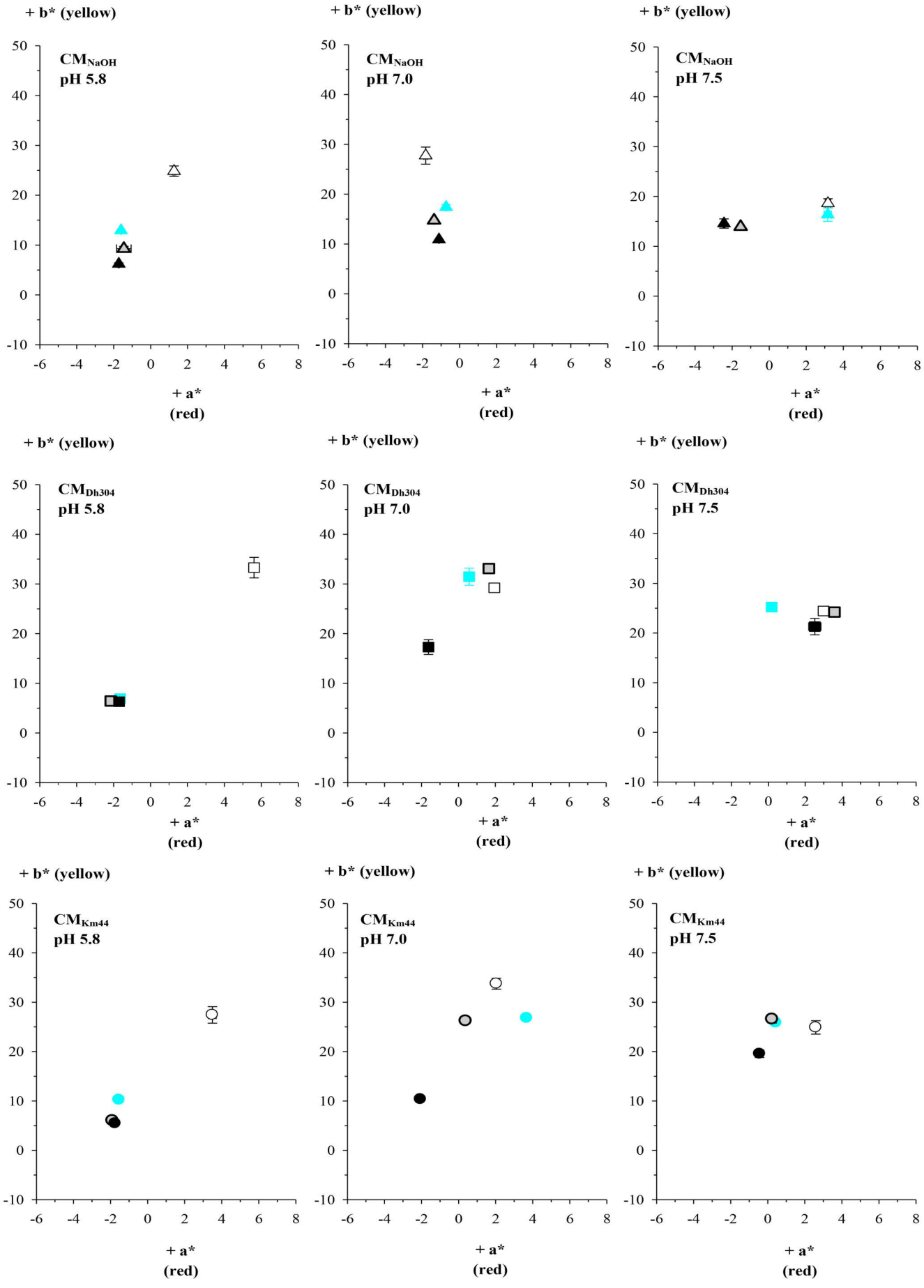


Fig. 1. Detailed positions of biofilms produced by *Arthrobacter arilaitensis* when projected in the CIE *L*a*b** colorimetric scale (bars indicate standard deviations). The concentration of additional NaCl (w/v) is presented as symbols following; □ = 0%, ■ = 2%, ■ = 4% and ■ = 8%.

Table 1 The h^o values of biofilms produced by *Arthrobacter arilaitensis* on cheese-based media deacidified by NaOH, *Debaryomyces hansenii* and *Kluyveromyces marxianus* after incubation for 4 weeks.

Initial pH of medium	Additional NaCl (w/v)	Type of medium		
		CM_{NaOH}	CM_{Dh304}	CM_{Km44}
5.8	0%	87.04 ± 1.95 ^{m}	80.53 ± 1.15 ^{b,c}	82.20 ± 2.20 ^{b}
	2%	100.26 ± 1.02 ^{a}	104.19 ± 0.39 ^{n}	98.37 ± 1.09 ^{a}
	4%	102.59 ± 0.83 ^{o}	104.46 ± 0.88 ^{p}	104.26 ± 1.28 ^{q}
	8%	105.53 ± 2.68 ^{r}	105.74 ± 0.73 ^{s}	105.25 ± 0.63 ^{t}
7.0	0%	93.76 ± 0.76 ^{u}	86.13 ± 0.72 ^{d,e}	84.80 ± 1.67 ^{d}
	2%	92.44 ± 1.43 ^{i}	87.69 ± 2.13 ^{v}	85.05 ± 2.31 ^{w}
	4%	95.35 ± 0.28 ^{j}	87.08 ± 0.13 ^{g}	89.13 ± 0.13 ^{g,h}
	8%	95.12 ± 0.18 ^{k,l}	95.18 ± 0.38 ^{l}	100.35 ± 1.25 ^{x}
7.5	0%	80.86 ± 0.67 ^{y}	83.04 ± 1.83 ^{z}	83.47 ± 0.74 ^{a*}
	2%	94.46 ± 1.16 ^{i}	88.51 ± 0.37 ^{e,f}	88.85 ± 2.23 ^{f}
	4%	96.33 ± 0.63 ^{j}	81.56 ± 0.56 ^{b*}	89.52 ± 1.39 ^{h}
	8%	99.55 ± 1.42 ^{k}	83.22 ± 2.05 ^{c*}	91.17 ± 0.33 ^{d*}

Values with a common letter including with or without symbol (*) do not statistically significantly differ (P>.001).
h^o of blank cheese-based agar was 102.85 ± 3.84.

Table 2 The saturation, C*, of biofilms produced by Arthrobacter arilaitensis on cheese-based media deacidified by NaOH, Debaryomyces hansenii and Kluyveromyces marxianus after incubation for 4 weeks.

Initial pH of medium	Additional NaCl (w/v)	Type of medium		
		CM_{NaOH}	CM_{Dh304}	CM_{Km44}
5.8	0%	24.53 ± 0.51 [b]	38.54 ± 1.48 [a]	37.12 ± 1.12 [a]
	2%	14.46 ± 0.13 [c,d]	7.71 ± 0.62 [m]	13.60 ± 1.72 [c]
	4%	9.66 ± 1.04 [n]	7.88 ± 1.08 [o]	6.79 ± 0.54 [p]
	8%	7.42 ± 0.25 [q]	6.71 ± 0.18 [r]	6.56 ± 1.44 [s]
7.0	0%	31.41 ± 0.69 [d]	31.24 ± 2.32 [t]	33.43 ± 1.17 [u]
	2%	17.10 ± 0.25 [v]	33.33 ± 2.31 [w]	26.91 ± 0.14 [e]
	4%	14.81 ± 0.34 [x]	35.17 ± 3.28 [y]	29.22 ± 2.58 [l]
	8%	12.53 ± 0.94 [g]	18.08 ± 2.42 [h]	11.15 ± 1.55 [g]
7.5	0%	25.37 ± 0.96 [b]	26.18 ± 1.05 [z]	29.50 ± 1.53 [a*]
	2%	24.17 ± 0.17 [b*]	30.19 ± 1.39 [f]	29.86 ± 0.94 [e,f]
	4%	23.70 ± 2.47 [c*]	31.89 ± 1.35 [k]	29.84 ± 1.85 [k,l]
	8%	22.16 ± 0.55 [d*]	17.46 ± 1.74 [h,i]	19.87 ± 0.97 [i,j]

Values with a common letter including with or without symbol (*) do not statistically significantly differ (P>.001).
C* of blank cheese-based agar was 5.38 ± 0.30.

The color attributes of *A. arilaitensis* biofilms versus time were measured during cultivation period on d 7, 14, 21 and 28. Among the same category i.e. initial pH and NaCl concentration, the overall changes during the cultivation on CM_{Dh304} and CM_{Km44} appeared to be dependent on these variables. The changes of color coordinate values obtained on all types of media at pH 5.8, excluding with 0% NaCl, were very slightly throughout the cultivation period, no significant difference between each time of the measurement ($P<.05$) (data not shown). On the other hand, when regarding *A. arilaitensis* biofilms on these deacidified media at pH 5.8 in the absence of NaCl, which displayed a yellow color between d 21 and 28, the development of color saturation between CM_{Dh304} and CM_{Km44} were similar, while there was strongly different between the two media and CM_{NaOH} (Fig. 2). The difference of the C^* values between CM_{NaOH} and the 2 others was obviously presented after d 7 and this occurrence remained until d 28. In contrast to CM_{Dh304} and CM_{Km44}, the C^* responses of *A. arilaitensis* biofilms on CM_{NaOH} slightly increased from the beginning until d 21, whose the values were not significantly different from those of the blank agar ($P>.05$) (data not shown). Then, between d 21 and 28, the change in its saturation was rapidly augmented, ranging from 8.99 to 24.53.

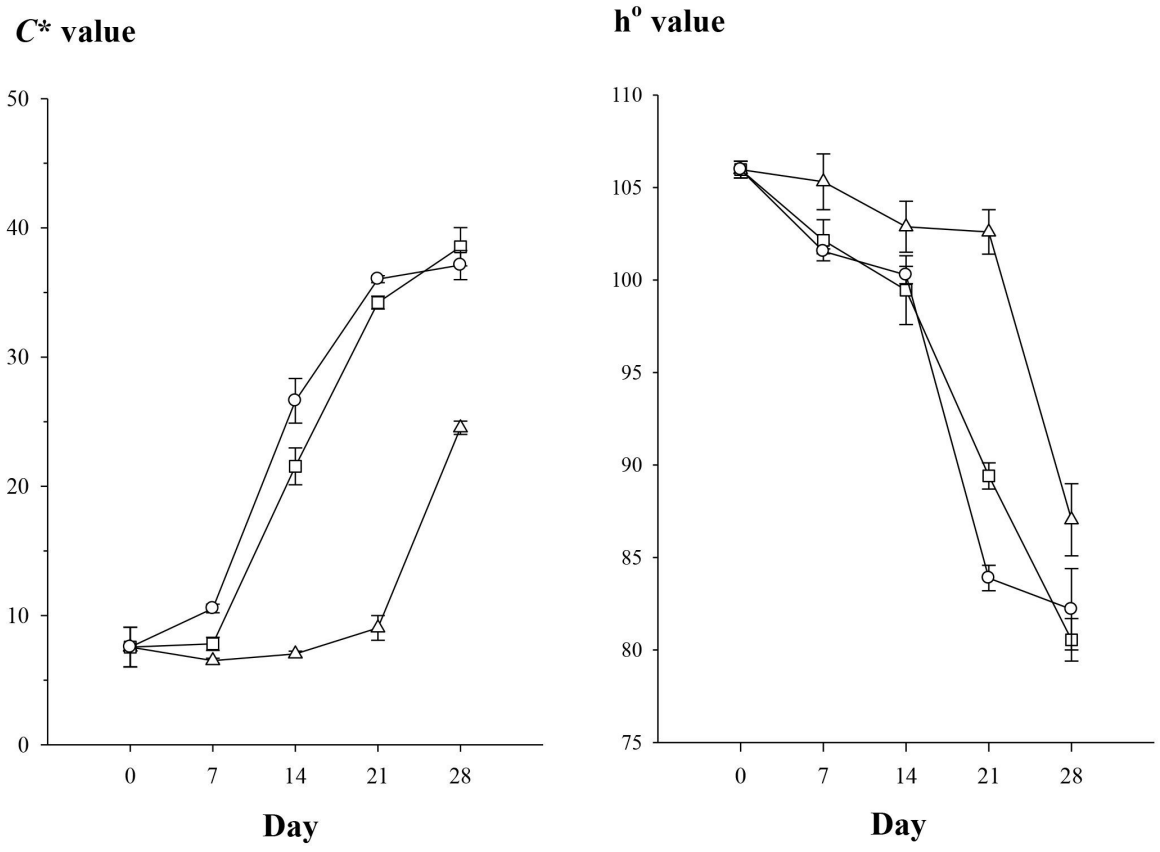


Fig. 2. Changes in color saturations and hues of *Arthrobacter arilaitensis* during growth on different deacidified cheese-based media at initial pH 5.8 without adding NaCl. △ = CM_{NaOH}, □ = CM_{Dh304} and ○ = CM_{Km44}.

However, the changes in these responses were different on the yeast deacidified media at pH greater than 5.8. The color development including chroma and hue of *A. arilaitensis* biofilms were extensively increased within 7 days of cultivation on CM_{Dh304} at pH 7.0 and 7.5 and CM_{Km44} at pH 7.5, containing NaCl varied between 0 – 4% (w/v), changing from white-cream to yellow, and then the increase became slow between d 7 and 28. Although almost of color development patterns by *A. arilaitensis* on CM_{Km44} were similar to those of CM_{Dh304}, the different forms of color evolutions were found at pH 7.0. The changes of saturation *C** provided by *A. arilaitensis* on CM_{Dh304} and CM_{Km44} , in the relation to pH and concentration of NaCl, versus time, are shown in Fig. 3.

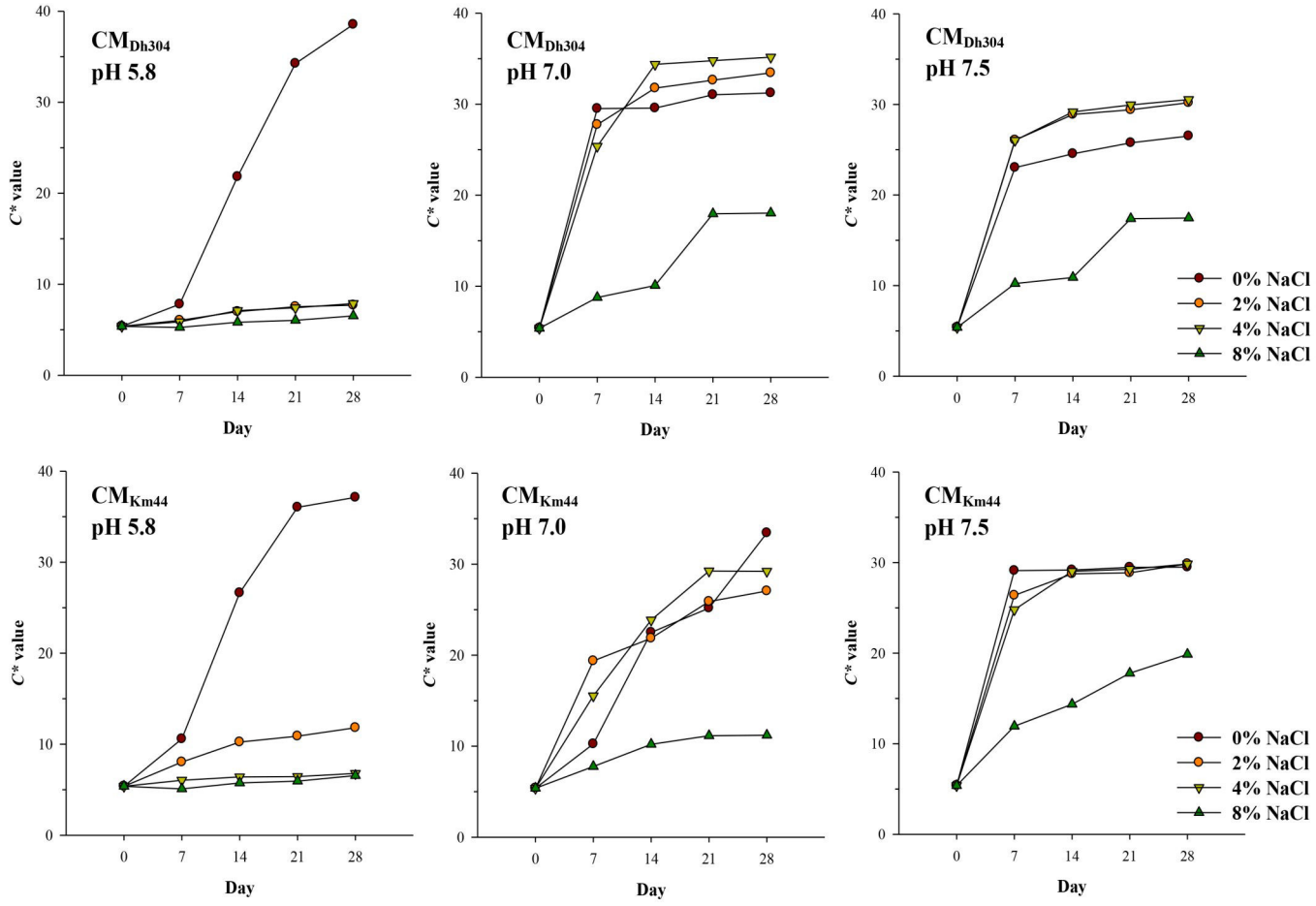
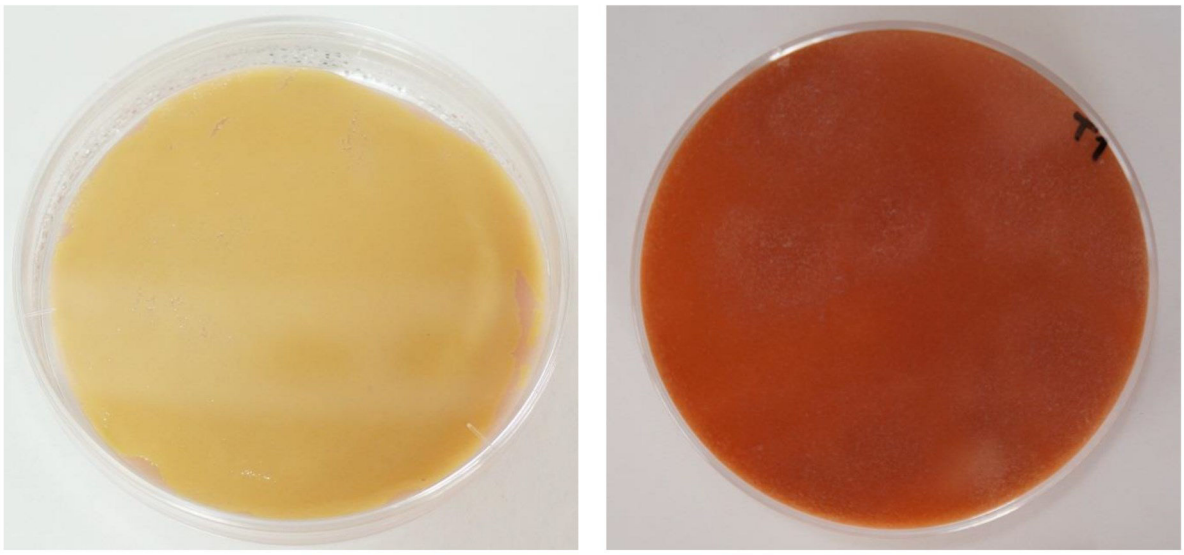


Fig. 3. Changes in *C** values of *Arthrobacter arilaitensis* biofilms during growth on CM_{Dh304} and CMKm44 at the initial pH 5.8, 7.0 and 7.5.

*L** values of *A. arilaitensis* biofilms on d 28 varied between 60.34 and 78.52, over all data observed on the present study (data not shown). Regardless of NaCl concentration, the highest lightness of the biofilms among each media was found at pH 7.0. Like the changes in color saturation, *L** values appeared to be rely on the factors controlled; furthermore, the period presenting changes in lightness was similar to those of chroma changes when using CM_{Dh304} and CM_{Km44}, adjusted pH 7.0 – 7.5.

Apart from the yellow pigments presenting in the biofilms produced by *A. arilaitensis*, it was likely that this strain also produced other pigment(s) diffusing inside the cultures due to a large number of cheese-based agar plates inoculated with this strain apparently displayed a pink color (Fig. 4). The pink color slightly appeared on certain plates during the first week of the incubation time; and then became more intense (red-brown colored) as well as more emerged in various treatments for media studied after d 14. When harvested on d 14, this occurrence particularly displayed on CM_{NaOH} at pH 7.5 containing 0 – 2 % (w/v) NaCl and on CM_{Dh304} and CM_{Km44} at pH 7.0 and 7.5 containing 0 – 4% (w/v) NaCl. However, the other cultures grown on different treatments; (i) CM_{Dh304} and CM_{Km44} at pH 7.0 and 7.5 with 8% (w/v) NaCl, and (ii) all typess of deacidified media at pH 5.8 without adding NaCl, showed the red-brown color on d 21 and 28, respectively.



A **B**

Fig. 4. Coloration by *Arthrobacter arilaitensis* Po102 on CM_{Dh304} at the initial pH 7.0 without adding NaCl, as an example for pink coloration observed on the cultures in this study. A = front view and B = rear view.

3.2.2 Changes in pH of Arthrobacter arilaitensis cultures during cultivation

Since the color development appeared not only on the top but also inside of the media inoculated with *A. arilaitensis*, pH of the cultures was measured instead of pH of the biofilms. When considering the whole experimental pH data of the present study, all controlled variables were likely to influence on the changes in pH of the cultures. The differences of pH changes between each type of deacidified media were appeared, especially between the media deacidified by additional NaOH and by deacidify-

ing yeasts, in addition, a large difference of pH changes during growth of *A. arilaitensis* was obviously displayed between pH 5.8 and the others. The comparison of changes in pH when using *D. hansenii* 304 and *K. marxianus* 44 in deacidification could categorized the patterns of these changes into several types depending on the initial pH of media. When the initial pH of deacidified media was 7.0 and 7.5, there were two major patterns of pH changes: (*i*) pH extensively augmented during the first week; and (*ii*) pH was stable, slightly increased or slightly decreased during the first 7 or 14 days, and then increased. The other patterns were observed on the initial pH 5.8: (*i*) pH increased between d 14 and d 21, (*ii*) pH remained constant over 28 days and (*iii*) pH decreased throughout the cultivation period.

In association with a pink color phenomenon on the cheese-curd media studied, the pH changes during growth showed to affiliate the occurrence of this color in *A. arilaitensis* cultures. Regardless of the NaCl concentration and the method used for deacidification, all of *A. arilaitensis* cultures would display a pink color when their pH increased to greater than 7.5 during growth (data not shown).

4. Discussion

During the last few years, *A. arilaitensis* have been identified as one important bacterial strain responsible for the coloration of smear-ripened cheeses; however, to our knowledge, only the study by Leclercq-Perlat *et al*. (2010) investigated the coloring capacity of this species on a variety of deacidified media preparing from cheese curds of Epoisses, Munster, Livarot and Reblochon. On the other hand, several previous studies have been emphasized *B.linens* on the cheese coloration in the relation to various factors such as pH, salt concentration, humidity, light, temperature and yeasts used in deacidification, as well as its interaction with other microorganisms isolated from ripened cheeses. The present study is the first investigation which emphasizes on the pigmentation of *A. arilaitensis* under the influence of initial pH, concentration of NaCl and deacidifying yeasts.

According to the data measured using spectrocolorimetry in the CIE *L*a*b** color system, diverse color characteristics provided by *A. arilaitensis* Po102 among three types of deacidified media. However, most of *A. arilaitensis* biofilms on CM_{NaOH} displayed h^o values ranking on greenish-yellow range, while hues of CM_{Dh304} and CM_{Km44} were set their color into reddish-yellow range. The h^o values on CM_{NaoH} in the present study were similar to a previous study on the pigments produced by a variety of *A. arilaitensis* strains, which showed the color characteristic of yellow *A. arilaitensis* strains with hues varying from 92.61 to 98.57 (Sutthiwong et al., 2014a). This could be explained by the resemblance of media used for each study, which both of them were adjusted the initial pH to 7.0 by NaOH. Besides, *A. arilaitensis* were reported to be sensitive to a type of media used for cultivation due to its color attributes were distinctly different when grown on media preparing from various cheese

curds (Leclercq-Perlat et al., 2010).

All controlled factors, i.e., initial pH, NaCl concentration and deacidifying yeasts appeared to affect the pigments produced by *A. arilaitensis*. When pH value initiated at 5.8, *A. arilaitensis* was able to produce pigments only in the absence of NaCl, whose viable cell concentration was significantly higher than those of cultures on the media added 2 – 8% (w/v) NaCl (data not shown). However, there were not significantly different of viable cell intensity between CM_{Dh304} and CM_{KM44} at this pH when added the same amount of NaCl, as well as their C^* and h^o values. These showed the relation between the pigment production of *A. arilaitensis* and its viable cell intensity; in consequent, it potentially indicated a strongly direct effect of initial pH on growth of *A. arilaitensis* Po102. The results of this experiment are consistent with the previous studies on the pigmentation of *B. linens* which reported a similar relation between pH, viable cell concentration and color intensity (Leclercq-Perlat et al., 2010; Masoud et al., 2003).

A comparison of color attributes provided by *A. arilaitensis* on CM_{Dh304} at pH values of both 7.0 and 7.5 apparently showed the differences between the cultures containing 8% (w/v) NaCl and the other ones containing 0 – 4% (w/v); meanwhile, there were less different among the media added 0 – 4% (w/v) NaCl, as well as the observation on CM_{Km44}. Resembling the data observed at pH 5.8, these differences probably depended on the viable cell concentration due to a number of viable *A. arilaitensis* was significantly highly lower when it grown on media added 8% (w/v) NaCl than when it grown on the other conditions (data not shown).

When considering the color of *A. arilaitensis* biofilms on CM_{Dh304} and CM_{Km44} at pH 7.0 and 7.5 containing 0 – 4% (w/v) NaCl, whose conditions were probably more suitable for the growth of *A. arilaitensis* in the present study, the color attributes including a^*, b^*, C^* and h^o were varied among these media; in addition, during the incubation time, the changes in their color were dissimilar, as well as their pH evolution. These results could refer to the relation between the yeast used in deacidification and the pigment production of *A. arilaitensis*. For preparing the media deacidified by yeasts, yeast cells were eliminated by pasteurization when pH of media reached the value required. By this reason, the impact of deacidifying yeasts on the pigmentation of *A. arilaitensis* possibly occurred from the difference in compositions of CM_{Dh304} and CM_{Km44}, not from biological interaction. Leclercq-Perlat et al. (2004) reported that *K. marxianus* required more lactose and lactate than *D. hansenii* for its growth during deacidification; therefore, ripening bacteria did not have sufficient lactate to possess energy for its growth, resulting in the concentration of biomass which directly related to the color presence. Furthermore, the ability of pigment production by *A. arilaitensis* appeared to depend on amino acid and caseinate composition of milk media (Bockelmann, Fuehr, Martin, Heller, 1997). According to Roostita and Fleet (1996), *D. hansenii* was able to produce several free amino acids (predominantly proline,

alanine, glycine, arginine and glutamic acid) and free fatty acid, during growth in milk, which probably affected the pigment production of *A. arilaitensis*.

In the present study, the development of pink/red-brown color evidently presented on *A. arilaitensis* on all types of deacidified media. No correlation between the red-brwon coloration and the concentration of NaCl, as well as the initial pH, was found on CM_{Dh304} and CMK_{m44}; however, after observing the changes in pH during growth, it could indicate that *A. arilaitensis* would produce the red-brown pigment(s) when pH values were greater than 7.5. Bockelmann (2002) reported that *A. nicotianae*, a yellow-pigmented bacteria isolated from German cheeses, was able to produce red-brown pigments when co-cultivated with *D. hansenii* and other bacteria under alkaline growth condition in shake liquid milk model system, as well as *A. nicotianae* in pure culture grown in liquid growth media containing casein hydrolysate. The same author demonstrated a possibility to use this species as a ripening culture for the typical red-brown Tilsit cheese. However, pink coloration (sometimes identified as pink-brown or dark-brown) has often been reported as a defect in a wide variety of smear-ripened cheeses, resulting in a reduction of cheese quality and a declination of consumers (Daly, MacSweeney, Sheehan, 2012). Many microorganisms as primary starter and secondary cultures have been implicated the pink discoloration in cheeses; however, to our knowledge, the pink pigments produced by *A. arilaitensis* have not yet been identified. Thus, a short experiment was performed to determine these pigments, in order to obtain the first information for further investigation on the role of pink pigmentation by *A. arilaitensis* in smear-ripened cheeses. The results showed that the pink pigments excreted by *A. arilaitensis* were soluble in water and the extracts exhibited fine structure in absorption spectrum with absorption maxima at 394, 497, 530, 567 and 620 nm; moreover, the extract fluoresced under UV light (data now shown), resembling the pigments of *A. nicotianae* which were water soluble and their extracts showed different absorbance spectra from carotenoid pigments of *B. linens* (Bockelmann et al., 1997). Based on a review on pigments produced by the genus *Arthrobacter*, these pink/red-brown pigments produced by *A. arilaitensis* are probably porphyrins (Sutthiwong, Fouillaud, Valla, Caro, Dufossé, 2014b). Several *Arthrobacter* species were reported to produce porphyrin pigments such as *A. hyalinus*, *A. globiformis* and *A. aurescens* (Kajiwara, Tokiwa, Takatori, Kojima, 1995; Kojima, Fujiwara, Mizutani, 1982).

5. Conclusion

Pigmentation of *A. arilaitensis* was sensitive to factors affecting its growth environment. A high concentration of NaCl showed the efficiency inhibited effect on the pigmentation and growth of *A. arilaitensis* Po102 in all types of deacidified media used for cultivation. In combination with initial pH

and NaCl concentration studied, deacidifying yeasts were obviously related to the pigment production of this *A. arilaitensis* strain. At pH 7.0 and 7.5, the color development on CM_{Dh304} was higher than on CM_{Km44}. Although, the highest color saturation of pigment produced by *A. arilaitensis* presented on CM_{Dh304} at pH 5.8 with 0% (w/v) NaCl, the highest average value of color saturation were observed on CM_{Dh304} at pH 7.0, presenting intense yellow. Besides yellow pigments presented as biofilm on the surface of deacidified media, the changes in pH during growth probably affected the pink/red-brown color development of *A. arilaitensis* Po102, which diffused inside cultured media. The results of this study have demonstrated a feasibility of controlling growth and pigment production of *A. arilaitensis*, in order to handle a quality of surface-ripened cheeses as well as to formulate a ripening starter culture which appropriates for a type of cheese.

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Author contributions

N. Sutthiwong and L. Dufossé designed the study. N. Sutthiwong performed the experiments. N. Sutthiwong and L. Dufossé interpreted the results. N. Sutthiwong drafted the manuscript. L. Dufossé revises the article critically.

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CONCLUSIONS

Pour un succès dans l'application d'*Arthrobacter arilaitensis* comme un acteur de la flore de maturation dans la fabrication de fromages à croûte lavée, une compréhension de la production de couleur par cette espèce est nécessaire. Dans cet objectif, plusieurs aspects impliqués dans la coloration de cette famille de fromages par *A. arilaitensis* ont été étudiés au cours de cette thèse dans un but de fournir des informations aussi complètes que possible pour expliquer la coloration de fromages à croûte lavée par cette bactérie.

Les caroténoïdes ont été clairement identifiés comme pigments produits par *A. arilaitensis* dans cette recherche, et la totalité des souches étudiées pourrait être classée en deux groupes, *i)* les souches productrices de caroténoïdes (soit 8 souches), et *ii)* les souches non pigmentées dans le cadre de nos conditions expérimentales (soit 6 souches). Tous les extraits pigmentés des souches productrices de caroténoïdes présentent des chromatogrammes HPLC identiques. Un modèle de pigmentation associée de la croissance a été appliqué pour expliquer la cinétique de production de pigments chez *A. arilaitensis*, dont les pigments de nature caroténoïde sont produits sous la forme de métabolites primaires qui agiraient comme agents protectants de photo-oxydation pour protéger les cellules contre les dommages de la lumière ou d'autres stress oxydatifs. Parmi les souches productrices de caroténoïdes, la diversité des niveaux de production de pigments est faible, ce qui constitue un résultat similaire à la caractérisation de la coloration à l'aide d'un spectrocolorimètre. Ces analyses en spectrocolorimétrie quantitative ont permis d'obtenir les caractéristiques de couleur de pigmentation d'*A. arilaitensis* en les exprimant dans le système colorimétrique CIE $L^* a^* b^*$. Huit souches, sur 14, ont présenté des cultures pigmentées jaune à jaune verdâtre, tandis que les autres ont présenté une couleur crème, pâle. Les positions détaillées de ces souches, projetées sur un plan (a^*, b^*), étaient très proches. Les résultats obtenus à partir de ces expériences, y compris les analyses UV-Vis/HPLC et la spectrocolorimétrie, ont été fortement liées les unes aux autres. Cela a conduit à indiquer que les souches *A. arilaitensis* des deux groupes, les souches productrices de caroténoïdes et les souches non pigmentées, peuvent jouer des rôles différents sur les fromages ; par exemple, les souches productrices de pigments caroténoïdes ayant une action conduisant à la coloration des fromages et à leur aromatisation, tandis que les souches non pigmentées peuvent être responsables uniquement de la saveur du fromage. Cette possibilité a déjà été décrite pour *Brevibacterium linens*.

Huit caroténoïdes différents ont été identifiés par HPLC-PDA-APCI-MS à partir de deux souches d'*A. arilaitensis* représentatives, jaune pigmentées. Quatre caroténoïdes, sur 8, ont très clairement été

caractérisés comme la décaprénoxanthine, la sarcinaxanthine, la 9-Z-décaprénoxanthine et la 15-Z-décaprénoxanthine, représentant les principaux caroténoïdes libres. Les autres caroténoïdes mineurs ont été éventuellement détectés en très faible quantité, et se composent de la sarcinaxanthine mono-glucoside pentaacétate, la décaprénoxanthine mono-glucoside, la décaprénoxanthine di-glucoside et la décaprénoxanthine-C16: 0.

Seules quelques études avaient été dédiées à *A. arilaitensis*, les pigments de cette bactérie n'avaient ainsi jamais été étudiés hormis leur présence sur la croûte des fromages de croûte lavée. À notre connaissance, les présents résultats constituent donc la première identification précise des pigments caroténoïdes produits par *A. arilaitensis*.

La lumière, un facteur physique qui a été précédemment rapporté pour influencer la pigmentation d'autres bactéries d'affinage tels que *B. linens* ou *Corynebacteriu*m sp., affecte aussi non seulement l'accumulation de pigments chez *A. arialitensis*, mais induit également des changements dans les caractéristiques de couleur observées par spectrocolorimétrie. Lorsque les souches productrices de caroténoïdes sont cultivées sous la condition de l'obscurité, la production de caroténoïdes était inférieure d'environ 3 fois à celle de la biosynthèse observée pour de la biomasse grandie dans la lumière. Considérant toutes les souches d'*A. arilaitensis* étudiées, trois groupes ont été décrits en fonction du comportement de coloration affecté par la lumière, (i) sensible positivement, (ii) sensible négativement, et (iii) insensible à la lumière. Toutes les souches pigmentées ont été classées dans un groupe sensible positivement à la lumière, changeant du jaune lors de la croissance à la lumière à crème-pâle en culture à l'obscurité. Bien que le rôle des caroténoïdes produits par *A. arilaitensis* n'a pas particulièrement été révélé, une fonction connue des caroténoïdes dans les micro-organismes est de servir d'antioxydants intégrés aux membranes, protégeant ainsi les cellules contre le stress d'oxydation.

En ce qui concerne l'hypothèse que plusieurs facteurs peuvent avoir un effet sur le développement de la couleur de bactéries d'affinage pendant la maturation des fromages, trois facteurs, c'est à dire le pH, le NaCl et les levures de désacidification, ont été employés comme imitant l'environnement du fromage.

Les changements dans les caractéristiques de couleur de la pigmentation par une souche productrice de caroténoïdes ont été révélés. Une variabilité des couleurs fournies par *A. arilaitensis* semblait dépendre des types de milieu désacidifiés, du pH et de la concentration en sel, en élargissant les notes de pâle à jaune intense. Le pH acide et une forte concentration de sel ont montré l'effet inhibiteur sur la pigmentation et la croissance d'*A. arilaitensis*. A un pH presque neutre, le développement de la couleur par la souche étudiée sur des milieux à base d'ingrédients-fromage désacidifiés par *D. hansenii* était plus élevé que sur le milieu utilisant *K. marxianus* pour la désacidification ; en outre, la valeur moyenne la

plus élevée de saturation de couleur a été observée à pH 7,0, montrant un jaune intense. Les résultats de cette thèse présentent ainsi une réelle amélioration des connaissances sur la pigmentation d'*A. arilaitensis,* sur son application dans l'affinage des fromages à croûte lavée, et peuvent conduire à développer une meilleure compréhension de la coloration de ce type de fromage lors de l'utilisation d'*A. arilaitensis* comme une partie de la microflore de maturation.

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